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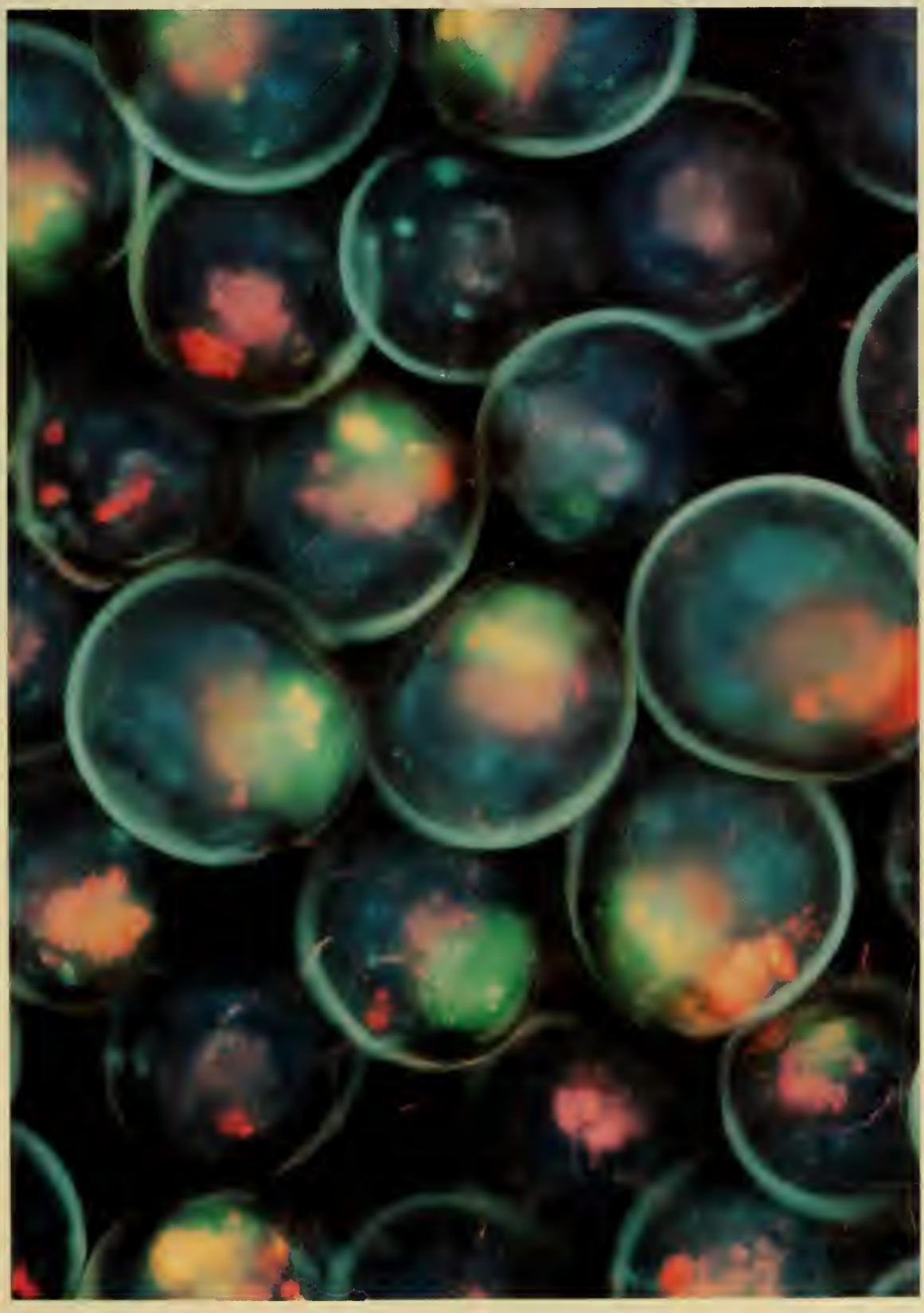

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IN MEMORIUM
JOHN CARL MEDCOF
1911–1997

J. Carl Medcof, a well-known and highly respected authority in the field of molluscan biology, oyster biology, and shellfish management in Atlantic Canada, died on 28 February 1997, in St. Andrews, New Brunswick. He was 86 years old.

Carl was born into a family with a strong academic background in Ruthven, Ontario in 1911, and the family moved soon after his birth to Toronto. His father, stepmother (his mother died when he was quite young), and uncles were all teachers. He received his elementary and secondary education mostly in Toronto and enrolled in the University of Toronto, where he received his B.A. in biology in 1932. He received an M.A. degree from the University of Western Ontario in 1934, undertaking a study of a snail, *Campeloma*, in a small river in southern Ontario as his thesis topic. Thus, began his lifelong interest in the field of molluscan biology. He received his Ph.D. degree from the University of Illinois in 1938, studying under Dr. H. J. Van Cleave, who was very influential in his early career. While at the University of Illinois, he was elected to the Phi Beta Kappa Society.

During his senior year at the University of Toronto and throughout his graduate years, Carl served as a teaching assistant. He maintained a keen interest in young people and always welcomed opportunities to talk with students and young scientists and encourage them in their work. He was a lifelong teacher, and many summer students benefited from his store of knowledge.

While an undergraduate, Carl began work as a summer student with the Biological Board of Canada, later the Fisheries Research Board of Canada. His first job was with investigations of Atlantic salmon. Subsequently, he worked as a summer student at Ellerslie, Prince Edward Island, where he came under the influence of Drs. Alfreda B. Needler for "red tides" and Alfred W. H. Needler, the director of the Station. Ellerslie was established to study oyster culture and foster development of the industry in the Canadian Maritimes. Thus, began Carl's enduring association with oyster research and the oyster industry of the Canadian East Coast. The title of his Ph.D. dissertation was "Studies on the larva of the Canadian oyster" and was focused on experiments with *Ostrea (Crassostrea) virginica* in the Bideford River, Prince Edward Island, Canada.

On completion of his Ph.D., Carl joined the staff of the Fisheries Research Board of Canada and was employed first at the Ellerslie Station, where he assumed responsibility for oyster research. He moved to the Biological Station in St. Andrews, New Brunswick in 1940, where he continued his work with oysters and also assumed responsibility for research work on all molluscan species including soft-shell clams, *Mya arenaria*, quahaugs, *Mercenaria mercenaria*, and sea scallops *Placopecten magellanicus* with his technician, Mrs. Esther Lord. Up until 1944, he moved with his family in the summers to Ellerslie and to St. Andrews in the winters.

Carl undertook a wide range of studies on oysters, including investigations on breeding to ensure a supply of juveniles for the industry, work to improve culture methods, and studies to improve harvesting and marketing. He maintained a close working relationship with the industry during his career, and much of his work focused on developing methods to improve it. He enjoyed working with people in industry and had their complete trust. Much of his work with oysters culminated with the publication, *Oyster Farming in The Maritimes*, which became a standard text for oyster culture in eastern Canada. This expertise took him to Cape Breton, Nova Scotia, where he worked with natives from the Eskasone Indian Band to grow oysters. He was also involved in experiments in the mid-1950s to transplant European oysters *(Ostrea edulis)* from France to the Bay of Fundy.

Carl worked extensively on the soft-shell clam with Mr. Stuart MacPhail from the Fisheries Research Board as well as with colleagues

from New England such as Mr. Dana Wallace. His clam work took him to eastern Nova Scotia, the Bay of Fundy, and the Gulf of St. Lawrence, where he worked with local harvesters to survey their areas and methods to increase production. He and Stuart MacPhail introduced the first water jet harvesters for soft-shell clams to the local industry and built prototypes for hand-held models as well as a vessel-equipped escalator harvester. Other clam species were studied as well. He and Ross Chandler from the St. Andrews Biological Station did the early biological work for the ocean quahaug clam fishery in southeast Nova Scotia.

Another major scientific contribution Carl made was in the field of shellfish toxins, particularly paralytic shellfish poisoning (PSP). As a result of his work and leadership, much of the history of PSP in the Canadian Maritimes was recorded and the etiology of outbreaks established. Results of this work led to establishment of a monitoring system for PSP to ensure that safe shellfish were marketed. Carl was the guest of honor at the Third International Conference on Toxic Dinoflagellates in 1985 and was awarded with a plaque in recognition of his contribution in this field. Later in his life, Carl said, "The most important work I did as a scientist was on resolving many paralytic shellfish problems. In addition I was able to work on methods of producing reliable forecasts about the expectancy of one of the major poisonous agents."

Carl also had a vision for the future. As early as the 1950s he was promoting the concept of aquaculture in marine production and was actively working on developing the protocols for producing soft-shell clams. He gave several media (including television) interviews and lectures on this topic. In addition to clams, he also predicted the development of the mussel culture industry in the Maritimes and an industry for sea urchin roe; something that is only just developing 25 years later.

Because of his expertise in shellfish, Carl was seconded to the Colombo Plan for 2 years in 1953 and 1955 and worked in Sri Lanka, assisting with development of invertebrate and other fisheries there. In 1955, he went to Europe to tour the various shellfish industries for information exchange with colleagues and for technology transfer. In the late 1960s, he undertook a similar trip to Japan with a group of Canadian scientists.

Carl retired from the Fisheries Research Board of Canada in 1973 and then spent a year in Australia, where he worked as a consultant to the shellfish industry through the University of Southern Australia in New South Wales. One project he undertook there was an investigation of the introduction of organisms via ballast water in ships, a subject that has become of great interest recently internationally.

Carl received numerous awards, both scientific and nonscientific, for the contributions he made during his lifetime. He was a long-standing member of the National Shellfisheries Association and served on the Editorial Board for the Proceedings and the Journal for many years. He was made an Honorary Member of the Association in 1973. He received a Centennial Medal from the Canadian Federal government in 1967 for his contributions to the Fisheries Research Board of Canada.

During his retirement, he taught a course in Marine Ecology at the Huntsman Marine Science Centre in St. Andrews. The course involved a rich mixture of basic biology, natural ecology, and the geological and paleological history of the Charlotte County area of New Brunswick.

Carl had a multitude of other interests and on his retirement began another career, recording the history of Charlotte County, New Brunswick, an offshoot of his previous hobby. He had long had an interest in the history of the area and in 1961 was a co-founder of the Charlotte County Historical Society, serving as its first president. He encouraged people to record what information they possessed of the area, and it was through his efforts as editor over a period of 21 years that an 12-volume collection of papers was published as *Contributions From The Charlotte County Historical Society*. As a result of his work with the Historical Society, Carl was presented with an Award of Merit from the Canadian Museums Association in 1981.

He was a devoted citizen of St. Andrews and took an active part in the affairs of the town, contributing to it in many ways over the years. During his lifetime, he was a Scoutmaster, Chairman of the Board of School of Trustees, a member of the local Kiwanis Club, and served as president. He was a member of the St. Andrews Anglican Church and published a history of that church. He served as vestryman, was an honorary church warden, and with his joy of singing, was a member of the church choir for many years. In 1987, the local Kiwanis Club selected him as their Man of the Year in recognition of his numerous contributions to the town.

Carl was a kind, gentle, humanitarian who brought out the best in people. He enjoyed working quietly, smoking his pipe (although more matches were burned than tobacco!), and he always had a package of dulse in his pocket, which he chewed and offered to anyone he met; he was one of the few people in the world who actually enjoyed chewing dulse! Those of us who were privileged to work under his direction will always remember the kind, thoughtful guidance and encouragement he gave us in our careers and his kind advice not only to be good scientists but to be good citizens. It was a joy to work and go on field trips with him, where we could enjoy his companionship and take part in long philosophical discussions on a wide range of biological and other topics.

Carl is survived by his wife of 55 years, Bessie, their three children, Susan, John, and Ranby, by three grandchildren, and by a great many people whose lives have been made more meaningful through their association with him.

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IN MEMORIAM
RUTH DIXON TURNER
1914–2000

Ruth Turner was born in Melrose, Massachusetts December 7, 1914. She attended Bridgewater State College, MA and graduated with a B.S. in 1936. She became a school teacher, teaching in both Bondville, Vermont and North Reading, Massachusetts before accepting the appointment of Assistant Director of Education at the New England Museum of Natural History (now the Boston Museum of Science). Ruth subsequently became Assistant Curator of Birds at the Museum, before moving to Vassar College as an Instructor in the Biology Department. During this same period she completed a M.A. at Cornell. By this time Ruth was an accomplished ornithologist, an interest that she maintained throughout her life. Indeed, it was her interest in birds that first lead her to the Museum of Comparative Zoology at Harvard. While serving as a volunteer in the Ornithology Department she met William J. Clench, Curator of Mollusks. Clench introduced Ruth to Dr. William Clapp, a pioneer in the study of marine wood borers, and in 1944 she moved to the William F. Clapp Laboratories in Duxbury, Massachusetts. It was here that her career as a malacologist became firmly established. She returned to Harvard University two years later to work with Clench.

Harvard remained her scientific home and a source of great pride to her throughout her career. One of her early field trips with Clench was to Cuba in 1949 to examine local terrestrial and marine mollusks. She received her Ph.D. from Radcliffe College, Harvard University in 1954. Her dissertation on the Teredinidae remains a standard work to this day. From 1954 through 1975 she served as Research Associate in the Department of Mollusks at the Museum of Comparative Zoology, Alexander Agassiz Fellow in Zoology and Oceanography, and Lecturer in Biology at Harvard. In 1976 she became Professor of Biology, Curator in Malacology at the Museum of Comparative Zoology, and joint editor of the scientific journal *Johnsonia*. Ruth received honorary D.Sc. degrees from New England College and Plymouth State College of the University of New Hampshire, and held honorary appointments at the Academy of Natural Sciences in Philadelphia, the Woods Hole Oceanographic Institution, the Gray Museum at the Marine Biological Laboratory at Woods Hole, Leigh University, CSIRO and the University of New South Wales in Australia, the University of Puerto Rico, and as an FAO Fellow in India. Ruth was honored by the Woods Hole Oceanographic Institution as a "Women Pioneer in Oceanography." On a lighter but no less important note, Ruth was named "Diver of the Year" by the Boston Sea Rovers, an educational society of which she was a very proud member. Ruth served terms as President of both the American Malacological Union and the Boston Malacological Club. She was a Honorary Life Member of the National Shellfisheries Association.

Ruth was a pioneer in the field of marine biodeterioration research, and enjoyed a long term relationship with the Office of Naval Research. This, combined with her work in invertebrate and larval ecology, took her to many corners of the globe including France, Belgium, Netherlands, England, Germany, Denmark, Puerto Rico, India, Pakistan, many locations in Australasia, South America, and the former Soviet Union. Although a leading researcher, she enjoyed teaching at all levels from special courses for public school teachers, to undergraduate and graduate teaching, to her gentle persuasion of fellow researchers to look at a problem or a data set in another light. Ruth's work also took her on many oceanographic cruises and to the depths of the ocean. On August 13, 1971, she became the first woman to dive in the deep submergence research vehicle ALVIN. This was the first of many dives and a deep sea career that included long term biodeterioration and species diversity work in the deep ocean (it was Ruth who explained why so little wood remained on the Titanic when it was found deep in the North Atlantic Ocean), and participation in multi-investigator cruises to the Galapagos Rift system.

Despite a career filled with discovery, innovation, and firsts, the most memorable component of Ruth's character that remains with the majority of people who met her is her warmth and friendliness, and her desire to show the excitement of science to all. Her love of science was effusive. She had a unique ability to share her science with audiences of all ages and skill levels. She was equally a superb teacher, illustrator, and practical scientist from fine work with the electron microscope to dissections of material from the field. Ruth taught and mentored many scientists at many levels, unselfishly giving of her time and energy to advance their careers. I consider myself fortunate to have enjoyed such direction from Ruth. Ruth shared much of her science through her publications, over 100 in all. Ruth worked actively until well after her 80th birthday. She left unfinished two major works, a monograph on her studies of deep sea borers and a comprehensive illustrated catalog of the pholads. Her colleagues have committed to finish these.

Throughout her career Ruth made unique contributions in malacology and deep sea biology. She was an internationally respected educator and researcher, an ambassador for marine and biodeterioration studies, and an outstanding role model for women in science. Ruth is survived by her sisters Winifred Garrity and Lina MacNeil. Ruth was predeceased by her brothers Henry and Arthur, and her sisters Jessie, Mary, and Frances. Ruth never married, but had a large extended family of colleagues and students. She will be sadly missed.

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Journal of Shellfish Research, Vol. 19, No. 1, 13–14, 2000.

Kenneth Kendall Chew
Honored Life Member

Dr. K. (Ken) K. Chew, is a recognised authority in the field of molluscan biology who has contributed extensively to invertebrate research and the continuing development of the shellfish industry on the west coast of North America. His contributions in the field of molluscan aquaculture are recognised world wide and his advice and consultation are frequently sought by industries in many countries.

Ken was born in Red Bluff, California in 1933 and received his elementary and secondary education there. While growing up, he worked in the family restaurant where he learned the fine art of Chinese cuisine. Those of us who have tasted Ken's cooking can attest to the fact that he mastered this fine art.

He obtained his B.A. from Chico State College in 1955 and then decided to attend the School of Fisheries of the University of Washington and become a fisheries biologist. Ken received a fellowship to begin graduate work on trout and salmon but Dr. Van Cleve sent him to the Washington State Department of Fisheries Shellfish Laboratory at Brinnon for the summer. He became involved in shellfish work at the lab and decided that his future lay with invertebrates, mainly molluscs, rather than fish and he entered the world of molluscan biology. He obtained his M.S. degree in 1958, studying the food preference of the Japanese oyster drill, and his PhD in 1962. The title of his PhD thesis was, "The growth of a population of Pacific oysters, *Crassostrea gigas*, when transplanted to three different areas in the state of Washington." His supervisor was Al Sparks, a former president of the National Shellfisheries Association.

After receiving his PhD, Ken joined the staff of the School of Fisheries at the University of Washington and has remained there since. He has held several positions at the School and has taught a variety of invertebrate courses. At present he is a Professor in the School of Fisheries. In 1989 he became the Director of the Western Regional Aquaculture Center (WRAC) which is one of five aquaculture centers designated by the U.S. Department of Agriculture to foster development of aquaculture in the United States. He resigned that position in 1996 to be the interim director of the School of Fisheries. He was then appointed Associate Dean, College of Ocean and Fisheries Sciences at the University of Washington in 1998, the position he holds presently. In addition to other duties, Ken is now busily engaged assisting with expansion of the College and seeking support for the College from industry.

Ken's research interests cover a wide spectrum that include shellfish biology and aquaculture, paralytic shellfish poisoning, and problems related to baseline ecological studies involving benthic intertidal and subtidal invertebrate communities. He has published over 100 papers on a wide range of shellfish subjects in scientific Journals, Technical Reports, Conference Proceedings, chapters of books and in columns of trade publications.

Teaching and maintaining a close rapport with students has been an important part of Ken's life and he has inspired many students to continue with studies in molluscan biology. During his career, about 100 students obtained graduate degrees under his supervision. In recognition of his outstanding teaching ability he received the 1993 Distinguished Undergraduate Teaching Award from the College of Oceans and Fisheries Sciences at the University of Washington.

Throughout his professional career, Ken maintained a close working relationship with the molluscan shellfish industry, particularly the industry in the Pacific Northwest. Much of his research and that of his graduate students focussed on finding solutions to problems to aid development of the industry. The present healthy state of the shellfish industry in the Pacific Northwest is due in a large measure to the efforts of Ken Chew and his co-workers. His talents and devotion to the shellfish industry were recognised when he was made Director of WRAC. In this position he devoted considerable time and energy testifying before Congressional Committees in Washington, DC on issues related to development of aquaculture.

Ken's influence in the shellfish industry has not been confined to the Pacific Northwest. He has provided advice and consultation to many countries throughout the world including; Australia, Canada, China, Chile, Japan, Thailand, Taiwan, Hong Kong and the Philippines. He has lectured on molluscan biology and culture in many countries. He was an invited guest lecturer in China and in 1987 was appointed for life as a visiting professor at Shandong College of Oceanography.

Ken has been actively involved with the National Shellfisheries Association since he first joined in 1958. He served on the Board, was Vice President from 1970–71, President from 1971–72 and served as an Associate Editor for the *Journal of Shellfish Research* from 1989–92. He has organized annual meetings held in Seattle. Another important function Ken has undertaken is to arrange Chinese Dinners at annual meetings. Many of us have enjoyed the fine cuisine and companionship that these evenings have afforded.

In addition to his involvement with the parent National Shellfisheries Association, Ken has played a major role to preserve and foster the association between the Pacific Coast Oyster Growers Association and the West Coast Section of the National Shellfisheries Association. From 1975–1990 Ken was the main reason this association survived and he devoted considerable time and energy into preserving this close association between industry and the scientific community of NSA. It is now a large and dynamic association and serves as an excellent forum for people from industry, government, and academia to come together and discuss shellfish work and problems. Many students have presented their first paper at these meetings. The present healthy state of this association is a monument to Ken's organisational skills and abilities.

As a result of his work and association with NSA, Ken was awarded the first David H. Wallace Award given by the Association in 1982 for his dedicated service in promoting research, understanding and co-operation among shellfisheries scientists, culturists, managers, producers and regulators. In further recognition of his contribution to NSA, he was elected to Honored Life Member in 1989.

Ken has been active in other organizations as well. He was a member of the Board of the World Aquaculture Society from 1973–76, President in 1977 and an associate editor of the Proceedings of the Society from 1985-89. He was editor for the North American Oyster Workshop that appeared as a special publication of the World Aquaculture Society in 1983. He received an Honorary Life Member award from the World Aquaculture Society in 1995. At present he is a columnist for Aquaculture Magazine.

Ken is an avid sportsman and, when time permits, relishes hunting and fishing. He is an excellent taxidermist and has mounted several species of birds he collected. He is a keen hand ball player and more than one meeting has been delayed so he could complete a game of hand ball.

Along with all his shellfish activities, Ken has found time to be an exemplary family man. He and his wife, Maegan, have raised four children and now have three grandchildren. They now have time to relax and enjoy their family and the view of Puget Sound from their wonderful house in Seattle. The shellfish world and NSA owe much to Ken Chew for his past contributions and his friends and co-workers know his influence will continue to be felt for many future years.

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Victor Samuel Stuart Kennedy
Honored Life Member

Dr. "Vic" Kennedy, a long time NSA member, Vice President (1988–1989), and President (1990–1991), was born in Ireland in 1942. Although long established in the U.S., he still maintains a Canadian, United Kingdom citizenship. His early education was at Sir George Williams University in Montreal, Canada, where he received his B.Sc. in zoology (1962). He continued graduate education at Memorial University at St. John's, Newfoundland, with a M.S. in fisheries biology (1964). Vic then entered the University of Rhode Island for his Ph.D. where Dr. Paul Saila was his major professor.

I first remember Vic by a phone call in 1967, whereby he introduced himself and said Dr. Saila suggested he call me because of our Chesapeake work in thermal ecology. He expressed an interest in doing the same with a shellfish species at our Chesapeake Biological Laboratory's field station at Hallowing Point about 20 miles up estuary from CBL. I invited him down and vividly remember our lunch at old famous Shorter's Restaurant in Benedict on the Patuxent Estuary. He impressed me with his quick mind, familiarity with the literature, and obvious intense interest in aquatic and shellfish ecology. I hired him on the spot and offered a pre-doc position. I called Dr. Saila, (an old friend that first befriended me as a graduate student at an American Fisheries Society meeting) afterward. I thought a graduate student accepted by Saul was surely good enough for me too.

Vic completed his Ph.D. on the role of temperature on the soft shell clam, *Mya arenaria*, in 1970 and has maintained a peripatetic professional career. After completing his Ph.D., he was visiting Assistant Professor at Chapman College in California where among other activities he spent two semesters traveling the world and teaching on board their "World Campus Afloat" vessel. He came back to Maryland in 1972–73 to continue thermal research, then left in 1973 for a Post-Doc fellowship at the University of Canterbury in New Zealand where he taught and completed research on mussels. In 1974, a Post-Doc was accepted at Newfoundland Biological Station in St. John's where he investigated the role of arsenic in a marine food web and on benthic soft bottom communities.

In 1976 he returned to the University of Maryland System's Horn Point Laboratory as an Assistant Professor and continued his teaching efforts as well as benthic ecology research. Again he left the Maryland area in 1983, and spent a sabbatical year as a W. F. Jones Fellow at St. Francis Xavier University in Nova Scotia where he continued his benthic research and taught a marine ecology course. In 1984 he returned to the Horn Point Laboratory, was promoted to Full Professor in 1986 and named Assistant Director in 1989. Again he left Maryland on a sabbatical and served as visiting Professor on board the *SS Universe Campus*, University of Pittsburgh ship, for the Semester at Sea Program. Vic has remained at Horn Point since! Throughout all this substantial traveling (40 countries), teaching

and current administrative duties, Vic has continued a vigorous research program dealing with shellfish reproduction and larval behavior, as well as crustacean and fish foraging behavior as reflected in the selected publications listing. His activities have covered both the littoral and sublittoral benthic habitats and communities. His long term interest in thermal ecology has now evolved into the global climate change arena.

Vic has over 45 journal refereed publications, over 10 chapters in books and conference proceedings and 5 written or edited books. He enjoys a special reputation for his publication efforts dealing with morphology, biology, ecology, and management history of the eastern oyster, *Crassostrea virginica.* A most important current activity is completing editorship of a 13 chapter book on blue crabs, which he has been working on with Dr. L. Eugene Cronin. (See Vic's In Memoriam to Dr. Cronin in *J. Shellfish Res.* 18(1):1–3, 1999). Another substantial service he has provided to the research and management communities are publications of 5 extensive bibliographies that have covered the world's literature in their respected areas.

Concerning professional societies, Vic has assumed numerous responsibilities over the years, including President of the Atlantic Estuarine Research Society, with the aforementioned National Shellfish Association and a governing board member of the Estuarine Research Federation. He has had editorial responsibilities for the *Transactions of the American Fisheries Society, American Malacological Bulletin,* and *Estuaries,* among others. Vic's service also includes numerous requests for research proposal reviews by NSF, Sea Grant, Hudson River Foundation, and the Smithsonian Institution. Beyond his usual numerous editing services, he regularly undertakes requests for reviews on books dealing with aquatic and coastal habitats and processes. This extensive editing experience he has translated to a very popular graduate course entitled "Scientific Writing and Communication" in which his last class had 38 students, an almost unheard of number in a graduate course.

In addition to the W. F. Jones Fellow honor he also won as NSA Thurlow C. Nelson award in his junior investigator days (1968), was noted for outstanding service by AFS, and given an Honored Life Member Award by NSA in 1995.

Vic shows no sign of slackening in his science enthusiasm and his very active and diversified professional involvements. Indeed, with his two children off in Canada, one in the creative arts and the other with her family working with the native Inuits on Baffin Island, I suspect he may even pick up the pace if his wife Debbie will allow!

Joseph A. Mihursky
Professor
Chesapeake Biological Laboratory

Journal of Shellfish Research, Vol. 19, No. 1, 17–18, 2000.

Sammy M. Ray
Honored Life Member

The scientific contributions of Dr. Sammy Ray to oyster disease research are widely acclaimed, due in no small part to the diagnostic method he developed to detect the disease agent *Dermocystidium marinum*. Dr. Ray was one of a handful of investigators in the early 1950's to explore this new oyster disease found in the Gulf of Mexico. Now the disease agent is called *Perkinsus marinus* and molecular techniques can be used to specifically diagnose the protozoan pathogen. Nonetheless, the highly reliable diagnostic technique developed by Dr. Ray is still the most widely used in oyster disease studies.

Dr. Ray was born in Mulberry KS, attended Mississippi Delta Junior College, Louisiana State University, and received his M.A. (Biology, 1952) and Ph.D. (Biology, 1954) degrees at Rice University in Texas. His postgraduate career began with the U.S. Fish and Wildlife Service as a Fishery Research Biologist and he joined the Texas A&M staff in 1957 at the Research Foundation Laboratory in Grande Isle, LA. He became an Associate Professor (1963) in Oceanography and Wildlife and Fisheries Science and was named Director of the Marine Laboratory at Galveston. As he reached Full Professor (1972), Dr. Ray was named Head of the Department of Marine Sciences. Since then he has held positions as Dean of the Moody College of Marine Technology and interim President of Texas A&M University at Galveston. Dr. Ray officially retired in 1990, but remains active as an advisor and coordinator of student programs and several community outreach programs.

Several academic honors have been awarded to Dr. Ray, including a Faculty Distinguished Achievement Award in Research at Texas A&M University at Galveston (TAMUG), the William Paul Ricker Award for Distinguished Faculty–Staff Achievement (TAMUG), a Distinguished Alumnus Award from the Mississippi Delta College, and a Piper Professor Award. He was awarded a lifetime honorary membership in the National Shellfisheries Association at the 1990 meeting in Maine.

Dr. Ray has been a reliable source of scientific information and advice for the State of Texas for many decades. He remains actively engaged in the interpretation of scientific knowledge for competent management decisions related to oyster and shrimp fisheries in the Gulf of Mexico. He has, over the last 10 years, participated in both the Joint Interim Committee on the Texas Shrimp and Oyster Industry and the Gulf of Mexico Fishery Management Council. Dr. Ray is a past chair of the Scientific and Technical advisory Committee for the Galveston Bay National Estuary Program and is a member of the Board of Trustees of the Galveston Bay Foundation.

Perhaps the most rewarding achievement of this exceptional career is the initiation of Sea Camp, "a hands-on marine adventure" for summer students aged 10–16, currently sponsored by TAMUG and the Texas Sea Grant College Program. Students attending the 5-day camps are given the opportunity to explore the Galveston Island area in research vessels, visit laboratory facilities and use scientific equipment to study marine organisms. Dr. Ray served as Director of the Sea Camp until 1993 and, in a similar capacity, is the Director of the Community & Youth Program for TAMUG. Dr. Ray and his wife Charlotte, an accomplished pianist now playing organ for the St. Luke's Episcopal Church, have four children and reside in Galveston.

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HABITAT AND REPRODUCTIVE BIOLOGY OF ANGELWINGS, *PHOLAS ORIENTALIS* (GMELIN)

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ABSTRACT The angelwing, *Pholas orientalis* (Gmelin) is indigenous to the coastal waters of the Provinces of Negros Occidental, Capiz and Iloilo in Central Philippines. They burrow into either muddy sand substratum in the littoral zone or compact bluish-gray muddy sand in the sub-littoral zone. They burrow to a depth of over 0.3 m and once extracted can never return. Specimens studied were invariably dioecious without apparent sexual dimorphism. Sexual maturity is reached at a shell length of 59 mm and 64 mm for males and females respectively. Each sexually mature individual possesses a gonad that is imbedded in the ventral side of the viscera. Both male and female gonads are arborescent in form and have the same coloration. Samples collected from Barotac Nuevo, Iloilo showed that the peak of spawning occurred from June through October and gametogenesis started in October.

KEY WORDS: *Pholas orientalis*, angelwing, reproductive cycle, gonad, spawning

INTRODUCTION

The angelwing, *Pholas orientalis* Gmelin, is one of the species of the family Pholadidae found in the Philippines. The other species are: *Barnea dilatata*, *B. manillensis*, and *Martesia striata*. *Pholas orientalis* is edible and is marketed either fresh or dried in Hongkong (haw chung), Malaysia (siput selat batu), Thailand (hoy pim), and Philippines (diwal) (Ablan 1938; Davidson 1976; Saraya 1982; Young and Serna 1982; Tokrisana et. al. 1985; Amornjaruchit 1988). It has a sweet, juicy and tender taste, making it one of the most highly sought bivalves in Central Philippines. However, indiscriminate harvesting has resulted in the depletion of most of the natural beds.

To date, the study of Ablan (1938) contains the only available information on the ecology and utilization of this species. To rehabilitate the depleted *P. orientalis* beds, detailed ecological and biological information is required. According to Rosell (1979), any attempt to manage the resource in the absence of baseline information is an exercise in futility. Thus this study was conducted to describe habitat and reproductive biology of the species.

MATERIALS AND METHODS

Habitat Adaptation

The study sites were Barotac Nuevo, Iloilo (122°47′N and 10°55′E) along Guimaras Strait and Roxas City, Capiz (122°45′N and 11°37′E) adjoining Pilar Bay, both in Central Philippines (Fig. 1). Ecological data from five random stations in each area were monitored during the study period. The grain size characteristics of the bottom sediments were determined after the procedure described by Buchanan (1971). Water temperature was measured using a calibrated laboratory thermometer and salinity was monitored using a refractometer. The pH of the water was determined using a pH meter. Monitoring of the environmental parameters was conducted from May 1994 to August 1995 at the Barotac Nuevo site and August 1996 to July 1997 in the Roxas City area.

Determination of Reproductive Biology

The specimens (n = 6–20) used for the study on reproductive biology were collected every month (May 1994 to August 1995) from the waters of the Barotac Nuevo site. Specimens were brought to the laboratory, where the size lengths were measured using a caliper, then shucked, and the gonads dissected. A portion of the gonad was examined with a Nikon Optiphot microscope to determine the sexes. The stages of maturity and gametogenic cycles were determined from histological preparations. Permanent mounts of the gonads were prepared following the modified Bell and Lightner (1989) method. The description of the gonadal stages were made following developmental stages for other clams (Jones 1981; Nash et al. 1986; Hesselman et al. 1989; Shafee and Daoudi 1991; Ponurovsky and Yakovlev 1992).

RESULTS

Habitat Adaptation

The characteristics of the two natural beds of Pholas orientalis in Central Philippines are shown in Table 1. *P. orientalis* from Barotac Nuevo were found to burrow in compact muddy sand (particles < 0.25 mm) covered with a thin layer of silt in littoral areas. No specimens were found in the sandy substratum of the littoral zone and on the deeper water. Few mangrove trees were found in the area, and seagrasses and macrobenthic algae were not observed. At Roxas City area, the angelwings occurred in the sublittoral areas to a depth of 8 m during the highest high tide, the bed being bluish gray compact muddy sand (coarse silt). No *P. orientalis* were found in the sandy mud bottom of the littoral zone. The natural bed was wholly devoid of mangrove trees and any rooted plants. In both locations, the angelwings burrowed in the substrata to a depth of about 0.3 m. On some occasions, burrows were found almost adjoining and may have met and crossed one another.

The trends of physico-chemical parameters (temperature, salinity and pH) in the two study sites during the period of observation are shown in Figure 2. The ambient water temperature in Barotac Nuevo ranged from 28 °C to 30 °C, and did not fluctuate widely. The lowest recorded temperature readings were in the months of December through February. At the Roxas City site, wider fluctuations in water temperature were observed (24 °C to 31 °C) with December to February being the coldest months. At both sites, the salinity readings were between the range of 30–35 ppt. A pH range

Figure 1. The natural beds (▬) of *P. orientolis* in Central Philippines. (●) Barotac Nuevo study site, and (✱) Roxas City study site.

from 7.8 to 8.2 was recorded throughout the study period at both study areas.

Reproduction

Sex

Out of total 147 sexually matured specimens that were used in this study, no hermaphroditic individuals were observed. Angelwings were dioecious without apparent external dimorphism. Once sexual maturity was attained, the single gonad was fused or imbedded on the ventral side of the visceral mass, extending from the anterior to the posterior part. Ripe male and female gonads had a creamy coloration, and were arborescent in form (Fig. 3A), whereas spent gonads were yellowish and flaccid (Fig. 3B). The epithelial walls of the viscera also reflected an almost creamy coloration, causing difficulty in sex differentiation and determination of the size of the gonad and gonadal index. Of the same 147 total gonads that were dissected, 78 (53%) were males, and 69 (47%) females.

TABLE 1.

Ecological information on the two natural beds of *Pholas orientalis* in Central Philippines.

Study Site	Habitat Type	Maximum Water Depth (m)	Substrate Type	Vegetation
Barotac Nuevo	intertidal	>1	muddy sand (particles <0.25 mm)	mangrove
Roxas City	sublittoral	8	compact bluish gray muddy sand (particles <1.00 mm)	none

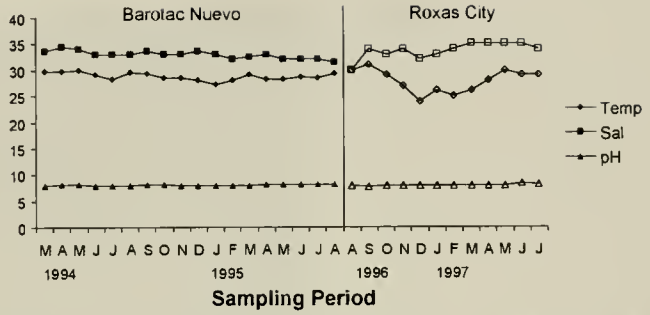

Figure 2. Some physico-chemical characteristics of water in the two natural beds of *P. orientalis* in Central Philippines.

Sexual Maturity

The specimens examined in this study ranged from 50–156 mm shell length. Most were found to be sexually mature. The minimum shell length of clams containing maturing gametes was 59

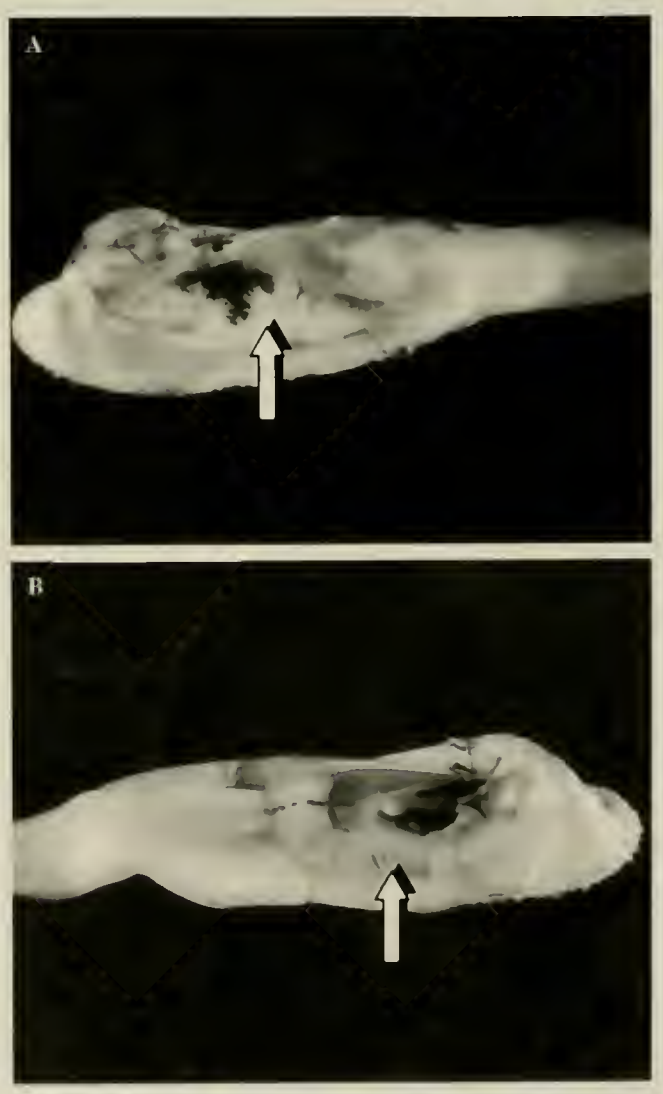

Figure 3. *P. orientalis* with (A) ripe gonad, and (B) spent gonad.

mm and 64 mm for males and females, respectively. The ages of the angelwings, however were not determined.

Gonadal Phase and Spawning

The gonadal state in both sexes was divided into five phases: early active, late active, ripe, partially spent and spent. The percentage occurrence of gonadal stages of male and female *P. orientalis* from Barotac Nuevo is shown in Fig. 4A and B, respectively.

Early active stage. Females follicles were empty and lined with small developing oocytes and oogonia. In males, few and loosely arranged spermatozoa were found in the center of the lumen of the follicle. These conditions occurred during the months of October to January. During this period, 14% of the male and 33% of the female angelwings population were in the early active phase.

Late active phase. In females, increased numbers of enlarging oocytes were freed in the lumen of the follicles. Oocytes were irregular in shape and had a wide range of sizes. In males, spermatocytes predominated the basal membrane of the follicle and numerous spermatids were found at the center of the follicle lumen. For both male and female gonads, about 17 to 60% were in the late active phase during the period December to May.

Ripe phase. In the female gonad rounded and ripe oocytes (with nucleus and nucleolus) were free in the lumen. In males, the gonad was predominated by mature spermatozoa in the lumen of the follicle; the acidophilic sperm tails formed lines radiating from the center of the follicle lumen. Specimens with ripe gonads were collected during the months of December to July. The percentage of ripe females ranged from 14.3 to 66.7, whereas males with ripe gonads ranged between 11.1 and 66.7.

Partially spent. Male gonads had spermatozoa missing in the central lumen of the follicle. Female gonads contained fewer ripe oocytes and appeared flaccid. Both types of gonad occurred in the months of May to October with percentage occurrence at 14.3 to 57.1.

Spent. Empty shrunken follicles were characteristic of spent gonads. This gonadal phase was observed from the months of June to October. By October, most of the angelwings had spawned.

DISCUSSION

P. orientalis is a commercially important yet poorly understood bivalve species found in Central Philippines. An early survey of Ablan (1938) showed that angelwings are indigenous to the coastal waters of Hinigaran, Pontevedra, Valladolid, and San Enrique in Negros Occidental, Philippines. A more recent survey indicated the presence of this species in the coastal waters of Barotac Nuevo toward San Dionisio in the Province of Iloilo and in Ivisan, Sapian, Panay, Pilar, Pontevedra and Roxas City all in the Province of Capiz (Fortes, unpublished). Apart from these areas no other site has been identified for the collection of the angelwings in the country. All the locations were within 3 to 100 miles at each other.

In this study, angelwings were found in either compact muddy sand or bluish gray muddy sand (with coarse silt) of the littoral or sublittoral zones. Ablan (1938) found the angelwings in a muddy

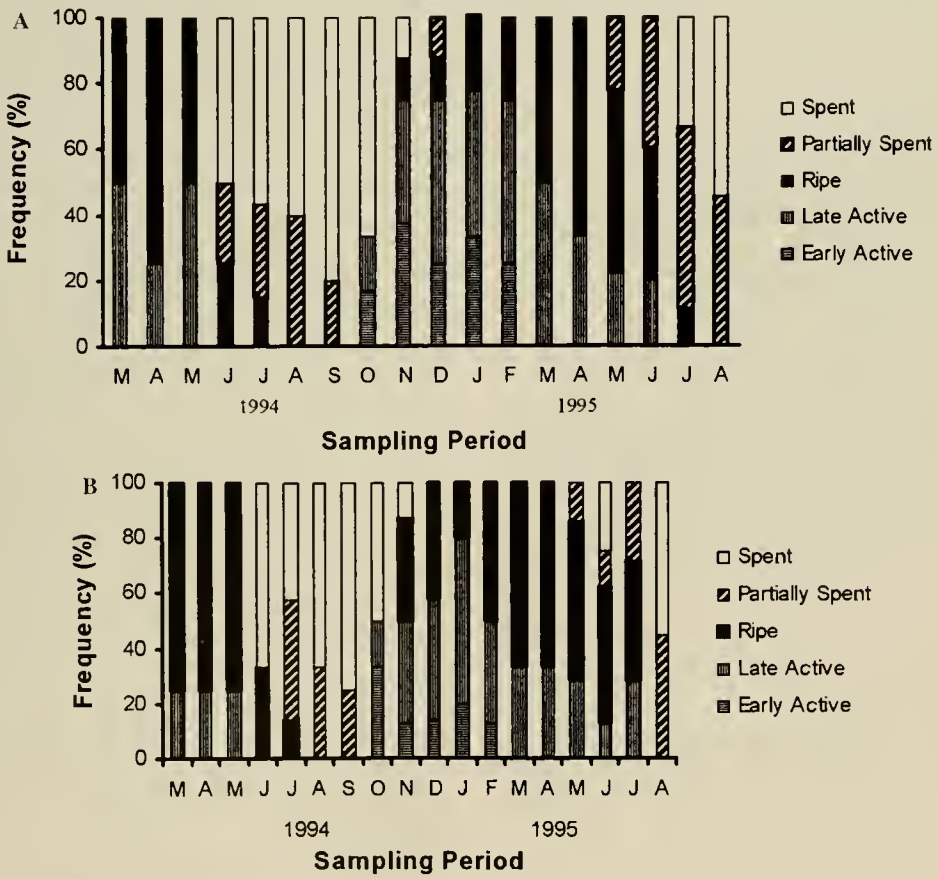

Figure 4. Reproductive cycle of *P. orientalis* in Barotac Nuevo, Central Philippines. Relative frequency of gonadal stages of (A) male, and (B) female between March 1994 to August 1995.

coastal land of Negros Occidental. A related species, *Cyrtopleura costata* has been observed to inhabit the sandy mud substratum in shallow waters from southern Massachusetts, USA, to Brazil (Turner 1954; Abbott 1974; Rios 1975). No clear explanation could be offered for the limited distribution of angelwings in the Philippines, and their contrasting ecological habitats (i.e., type of bottom sediments, water depth).

The pholads are capable of burrowing to a depth over 0.3 m (Ablan 1938; Allan 1959; this study). They live in the burrows for life (Allan 1959), and once extracted from their lodge they are unable to return. The burrowing ability is necessary to protect themselves from predators and the adverse effects of the physical environment as their shells are fragile. For *C. costata*, they begin burrowing after larval settlement, and recorded effective burrowing size was at a mean shell length of 11.7 mm (Gustafson et. al. 1991). Larger individuals (> 15 mm) of the same species were unable to rebury and had to be manually placed beneath the sediments during field-planting. However, effective burrowing size for the *P. orientalis* is not known yet.

Angelwings seem to have an extended annual breeding cycle, where initiation of gametogenesis begins almost after spawning. It was observed that sizes of specimens had no effect on the timing of gametogenesis. Small or large specimens, as long as they are sexually mature exhibited almost simultaneous gametogenesis. Gametogenesis was observed in the months of October to January. The month of October was period when most of the clams were partially spent or spent. The peak of spawning occurred in the months of June and October, at onset of the rainy season in the Philippines. Chanley and Andrews (1971) reported the spawning period from May through September for *C. costata* from Virginia, USA, whereas specimens from subtropical Florida were ripe in the summer months of June through August. The cyclical reproductive pattern observed in *P. orientalis*, however, cannot be definitely and clearly related to temporal changes in temperature and salinity. The lack of effect of temperature on the reproductive cycle was similarly observed on venerid clams like *Megapitaria aurantiaca, M. squalida*, and *Dosinia ponderosa* from Bahia Zihuatanejo, Mexico (Baqueiro and Stuardo 1977 cited by Garcia-Dominguez et al. 1998), and the giant reef clam *Periglypta multicostata* in Isla Espiritu Santo, Baja California Sur, Mexico (Garcia-Dominguez et al. 1998).

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INFLUENCE OF DIET ON SURVIVAL, GROWTH, AND PHYSIOLOGICAL CONDITION OF FINGERNAIL CLAMS *MUSCULIUM TRANSVERSUM*

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ABSTRACT The effects of diet and laboratory holding time on survival, growth, and physiological condition of fingernail clams *Musculium transversum* were evaluated in a 112-day study. The diets included a commercial oyster diet, a suspension of commercial rabbit pellets, a suspension of fine, organic-rich sediment, and a complete sediment renewal every 14 days. Sediment and clams were obtained from a relatively uncontaminated site in the Upper Mississippi River. The experimental design consisted of 18 370-mL beakers per diet, each containing 5 cm of surficial sediment and 15 clams. Survival of clams was measured daily in each unit. Three units from each diet were randomly removed on days 7, 14, 21, 28, 56, and 112, and clams were measured for shell length. Glycogen and cellulase activity were measured in composite samples (5 clams per sample) at each of the six time intervals. Cellulase activity did not vary among diets or with time. Survival, growth, and glycogen varied significantly among diets, and glycogen concentrations varied with time, regardless of diet. Clams exposed to the two sediment diets were 2.4 times more likely to survive than clams exposed to the commercial diets. Survival of clams in all diets exceeded 80% through day 21. Although clams maintained an acceptable survival rate for 21 days, their physiological condition was compromised much earlier, given that glycogen reserves were reduced by 14–54% after only 7 days. Thus, laboratory tests with fingernail clams should include physiological measures, in addition to survival, to ensure that clams are in suitable condition before and during testing.

KEY WORDS: Diet, *Musculium transversum*, survival, growth, biomarker

INTRODUCTION

Fingernail clams are an important component in the benthic invertebrate community of many large rivers and, in the Upper Mississippi River, have undergone periodic, pronounced declines in abundance in recent decades (Wilson et al. 1995). For example, densities in Pool 19 (near Keokuk, IA) averaged 32,000/m^2 in 1985 and progressively declined to 0 in 1990, and river-wide recovery has been slow. Toxicity of bulk sediment or pore water has been suggested as a factor contributing to the decline in fingernail clams in the river (Wilson et al. 1995). In particular, concentrations of un-ionized ammonia in sediment pore water from the Upper Mississippi River often exceed concentrations demonstrated to inhibit growth of fingernail clams in laboratory studies (Frazier et al. 1996). To assess these and other potential causes of the decline in abundance requires that clams be collected from the field, held in the laboratory, and tested through controlled experimentation. However, information on the relative condition of clams during long-term holding and its effect on the outcome of laboratory tests is lacking (Naimo et al. 2000).

The physiological condition of an organism is dependent upon its nutritional status (Lanno et al. 1989, Foster et al. 1993). Yet, the importance of nutrition as a factor modifying physiological condition has been largely overlooked. Data on how the condition of an organism responds to its nutritional status are critical for understanding the importance of diet as a variable in designing experimental studies with benthic organisms.

Recently, physiological indicators of condition such as glycogen concentration and cellulase activity have been used to assess the relative health of bivalve mollusks (Hemelraad et al. 1990, Haag et al. 1993, Farris et al. 1994, Naimo et al. 1998). Glycogen is the most readily available storage form of glucose in many animals, including freshwater mussels. As such, glycogen concentrations have been used successfully as an indicator of physiological condition in unionid mussels after exposure to contaminants (Hemelraad et al. 1990) and after infestation by zebra mussels (Haag et al. 1993). Similarly, cellulase activity is an indirect measure of feeding because it measures the rate of breakdown of complex sugars into simple molecules (Farris et al. 1988). Extensive use of cellulase activity in monitoring programs for molluscs has shown that responses at the biochemical level can be measured where pollutants or stress first exert their effect (Beeby 1993, Milam and Farris 1998). In these studies, the predictive capability of the enzyme assay has been compared with extensive testing of more traditional biological endpoints in toxicity assessments. Controlled laboratory and field exposures have provided evidence that reductions in enzyme activity are related to the eventual survival of the animal and to more subtle changes that occur in filtration, growth, and bioaccumulation rates (Farris et al. 1994, Milam and Farris 1998).

We examined survival, growth, and physiological condition in clams provided different food sources in a 112-day laboratory study. Our specific objective was to evaluate the effect of diet on the survival, growth, and physiological condition of fingernail

clams *Musculium transversum* (Say 1829). Furthermore, because we were interested in the transferability of these data to standardized tests with benthic invertebrates, we examined differences in survival, growth, and physiological condition between clams fed two commercially available diets (easily reproducible, but a non-indigenous diet) and two diets containing sediment (not as reproducible, but more indigenous).

MATERIALS AND METHODS

Experimental Design

We obtained about 600 fingernail clams with a Ponar dredge from Pool 13 of the Upper Mississippi River for use in the laboratory test. During collection, clams were placed in ice chests containing sediment and water from the river. The water in the ice chests was aerated and its dissolved oxygen content was measured at 30-min intervals to maintain concentrations above 60% of saturation. To obtain an estimate of the physiological condition of clams at this point in time, we obtained an additional 15 clams, placed them on dry ice in the field, and stored them at −84 °C in the laboratory before analysis of glycogen concentration and cellulase activity.

The uppermost 5 cm of sediment from a single sampling site in Pool 7 of the Upper Mississippi River (Lake Onalaska, river mile 704.5) that contained an abundant fingernail clam population was obtained with a van Veen dredge. Sediment was placed into 4-L glass jars, held on ice, transported to the laboratory, and stored in a refrigerator for no more than 5 days before the start of the test. Three subsamples of homogenized sediment (each 20–25 g wet weight) were analyzed to describe textural composition (Guy 1969, Plumb 1981) and volatile matter content (American Public Health Association et al. 1992). Sediments averaged (mean ± 1 standard error [SE]) 4 ± 0.2% sand, 54 ± 2.4% silt, 42 ± 1.8% clay, and 7.8 ± 0.9% volatile matter.

The experimental unit was a 370-mL beaker. All experimental units were placed into one of two 900-L water baths (3 m length × 0.8 m width × 0.4 m height). Each water bath was partitioned lengthwise with Plexiglas to provide four compartments, one for each diet. Eighteen experimental units were randomly allocated into each compartment. A temperature of 17 ± 2 °C was maintained with submersible quartz heaters. About 24 h before the addition of clams, 184–188 g of surficial sediment (about 4–5 cm) and 200 mL of well water from the Upper Midwest Environmental Sciences Center were added to each experimental unit. On day 0, we randomly allocated 15 clams, each measuring 4–6 mm in shell length, into each experimental unit.

We measured the temperature, pH, and dissolved oxygen of the overlying water every Monday, Wednesday, and Friday in five randomly selected experimental units in each diet. Because fingernail clams are particularly sensitive to un-ionized ammonia (Hickey and Vickers 1994), we measured concentrations of total and un-ionized ammonia in three randomly selected experimental units every 14 days (Frazier et al. 1996). On days 7, 14, 21, 28, 56, and 112, clams from three randomly selected experimental units from each diet were sieved from test sediments, counted, recorded as dead or alive, measured for shell length to the nearest 0.1 mm, and stored at −84°C for later analysis of glycogen concentrations and cellulase activity. Glycogen concentrations (Naimo et al. 1998) and cellulase activity (Farris et al. 1988) were measured on composite samples containing five individuals from each experimental unit. Glycogen concentrations were reported as mg/g wet weight, and cellulase activity was expressed as a product (exocellulase activity times endocellulase activity in [units/g dry weight]^2). One unit of the enzyme is defined as the amount of enzyme required to liberate 1 mg of reducing sugar equivalent to that of glucose per hour with carboxymethylcellulose as a substrate.

Diet and Ration

Clams were fed one of four diets daily; two were commercially available diets, and two were formulated with sediments from the Upper Mississippi River (sediment diets). The commercial diets included an oyster diet, which was a mixture of two marine diatoms (50% *Thalassiosira pseudoana* and 50% *Skeletonema sp.*) fed at a rate of about 7μL/clam/day (8–10 × 10^9 cells/mL; Pacific Oyster Diet B, Coast Seafood Company, Quilcene, WA). The second commercial diet was a suspension of Kaytee® rabbit feed, with pellets made largely from alfalfa, fed at a rate of 2.5 mg/clam/day. The two sediment diets contained organic-rich sediments from relatively uncontaminated areas in the river and were the same sediment used as the substrate in all experimental units. One was a suspension of fine sediment fed at a rate of 2.5 mg/clam/day, and the other was a complete sediment renewal every 14 days.

The oyster diet, rabbit pellet diet, and suspended sediment diet were prepared about 2 days before the start of the experiment. The oyster diet comes in liquid form and was kept refrigerated. The rabbit pellet and the suspended sediment diets were prepared by blending 38 g of rabbit pellets or sediment with 400 mL of well water in a commercial blender for 5 min. The contents of the blender were transferred into a 1,000-mL volumetric flask and filled to the meniscus with well water. This process was repeated until we obtained 32 140-mL bottles of each diet. Once a week, one bottle of food for each diet was removed from a −20°C freezer and placed into a refrigerator; the quantity of food in each bottle was sufficient to feed all clams receiving those diets for 1 wk. Clams in the sediment-renewal diet were sieved from test sediments every 14 days, and another aliquot of sediment was replaced into each experimental unit. Sediments for this diet were the same sediments that were obtained at the start of the test, stored in a refrigerator until needed.

Statistical Analyses

Survival of clams was assessed by daily counts of dead shells on the sediment surface. In addition, at the six time intervals in which clams from three beakers were removed for physiological measurements, we also made direct mortality estimates; these data allowed us to check the accuracy of the daily mortality counts. Because these two estimates agreed more than 90% of the time, analyses of survival rate were performed on daily survival counts. We used the Cox proportional hazards model to determine whether survival rates of clams varied among diets (Cox 1972). To test for differences in survival between the commercial and sediment diets, we used the Wald test of equality (Parmar and Machin 1995).

We analyzed growth, glycogen concentrations, and cellulase activity with analysis of covariance (ANCOVA), with time in the laboratory as the covariate. Because most clams did not survive after day 56, statistical analyses were only conducted until day 56. Orthogonal contrasts were used to compare differences in growth and physiological condition between the commercial and sediment diets when the ANCOVA was significant. We did not record the shell length of each clam on day 0; instead, we ensured that all

clams ranged from 4 to 6 mm in length. Because shell length did not differ among diets at day 7 ($P = 0.21$), subsequent analyses were performed on shell length measures from day 7 through day 56. A type I error α of 0.05 was used to reject all null hypotheses.

RESULTS

The quality of the overlying test water was similar among diets (Fig. 1). For example, grand means (averaged over all diets and time periods) ranged from 15.4°C to 15.7°C for temperature, 8.2 to 8.3 for pH, and 9.7 to 9.8 mg/L for dissolved oxygen. Concentrations of total (range, 0.03–0.13 mg/L) and un-ionized (0.002–0.008 mg/L) ammonia were well below concentrations that adversely affect fingernail clams in laboratory exposures (Sparks and Sandusky 1981).

The survival rate of fingernail clams varied significantly among diets ($P = 0.0001$). Survival rates were lowest in clams fed the oyster diet, whereas survival was highest in clams receiving the sediment-renewal treatment (Fig. 2). For example, survival averaged 44% in the oyster diet, 66% in the rabbit-pellet diet, 73% in the suspended-sediment diet, and 84% in the sediment-renewal diet at day 56. By day 112, only 6% of the clams in the sediment-renewal treatment were alive, and none survived in the other three dietary treatments.

Survival was significantly greater in clams provided the sediment diets, relative to the commercial diets ($P = 0.0001$). After 56 days in the laboratory, for example, survival of clams fed the sediment diets averaged 79%, whereas survival averaged 55% in clams fed the commercial diets. However, there was little difference in survival of clams among diets early in the test; survival of clams in all diets exceeded 80% through 21 days of exposure. A unique feature of the proportional hazards model is the ability to calculate a risk ratio, or the estimated hazard of surviving in one diet versus another. For example, clams provided the oyster diet were 1.9 times more likely to die than clams fed rabbit pellets (Table 1). Additionally, clams fed the oyster diet were almost 5 times more likely to die than clams in the sediment-renewal treatment. Furthermore, clams fed the commercial diets were 2.4 times more likely to die than clams fed the two sediment diets.

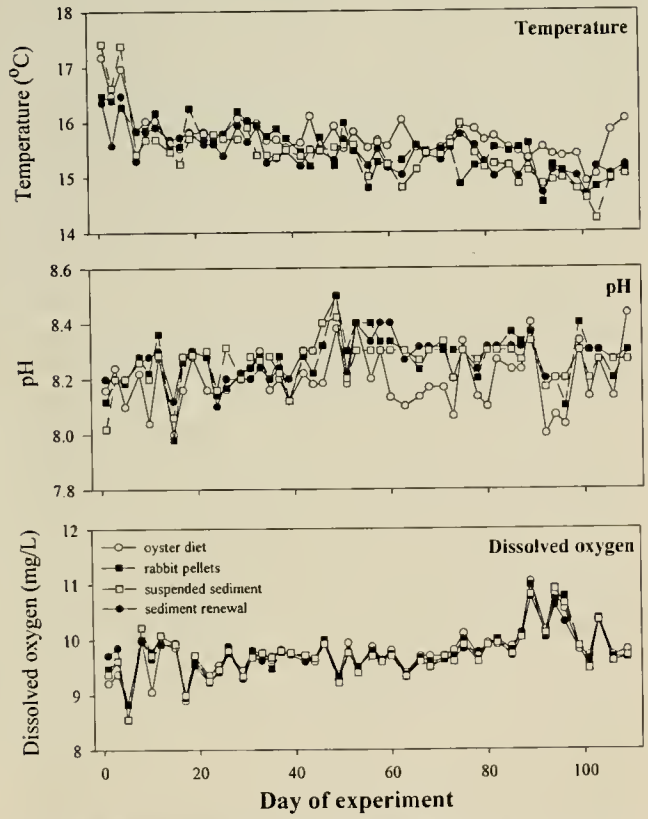

Figure 1. Mean temperature, pH, and dissolved oxygen in overlying test water from five randomly selected experimental units containing fingernail clams *Musculium transversum* fed one of four diets daily for 112 days.

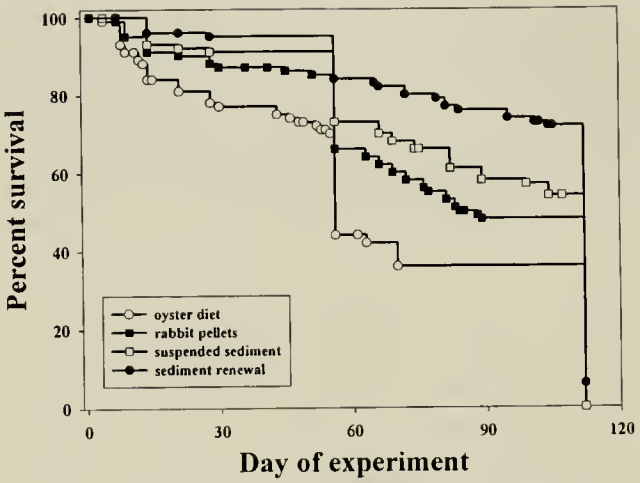

Figure 2. Survival of fingernail clams *Musculium transversum* fed one of four diets in a 112-day laboratory test.

The shell length of fingernail clams also varied significantly among diets ($P = 0.02$). Clams receiving the sediment-renewal treatment were significantly larger than clams in the other three dietary treatments. For example, clams in the sediment-renewal

TABLE 1.

Estimated probability values, risk ratios, and upper and lower 95% confidence limits from the survival rate analysis in fingernail clams fed four different diets in a 112-day laboratory experiment.

Contrast	*P* Value	Risk Ratio	Lower 95% Confidence Limit	Upper 95% Confidence Limit
Oyster diet, suspended sediment	0.0001	2.6	1.9	3.5
Rabbit pellets, suspended sediment	0.1124	1.3	0.9	1.9
Sediment renewal, suspended sediment	0.0120	0.5	0.3	0.9
Oyster diet, rabbit pellets	0.0001	1.9	1.9	2.0
Oyster diet, sediment renewal	0.0001	4.8	4.0	5.8
Rabbit pellets, sediment renewal	0.0002	2.4	2.2	2.8
Commercial diets, sediment diets	0.0001	2.4	1.9	3.2

The risk ratio is the estimated hazard of surviving in one diet *versus* another diet; for example, clams fed the oyster diet were 2.6 times more likely to die than clams fed the suspended-sediment diet.

treatment averaged 4.8 mm in length over the 56-day duration, whereas clams in the other three dietary treatments ranged from 4.3 to 4.4 mm. Furthermore, the size of clams did not differ between clams provided the commercial and sediment diets (P = 0.50), nor did shell length vary with time in the laboratory (P = 0.23; Fig. 3a). At day 7, clams ranged in length from 4.2 to 4.8 mm and at day 56, they ranged in length from 4.5 to 4.8 mm.

Glycogen concentrations in clams varied significantly among diets (P = 0.049; Fig. 3b). In particular, glycogen concentrations differed between the commercial and sediment diets (P = 0.02). For example, mean glycogen concentration was 3.5 mg/g in clams fed the oyster diet and 4.1 mg/g in clams fed the rabbit pellets. In contrast, glycogen concentrations averaged 2.8 mg/g in the suspended-sediment diet and 3.0 mg/g in the sediment-renewal treatment. However, glycogen concentrations declined significantly with time in the laboratory, regardless of diet (P = 0.0001). For example, glycogen concentrations in clams in the sediment-renewal treatment averaged 4.6 mg/g at day 7 and had declined to only 2.2 mg/g by day 56. Moreover, because there was no diet*time interaction (P = 0.49), the response of glycogen with time was similar among diets. For reference, glycogen concentrations averaged 5.4 ± 0.5 (SE) mg/g in clams when they were removed from the Mississippi River.

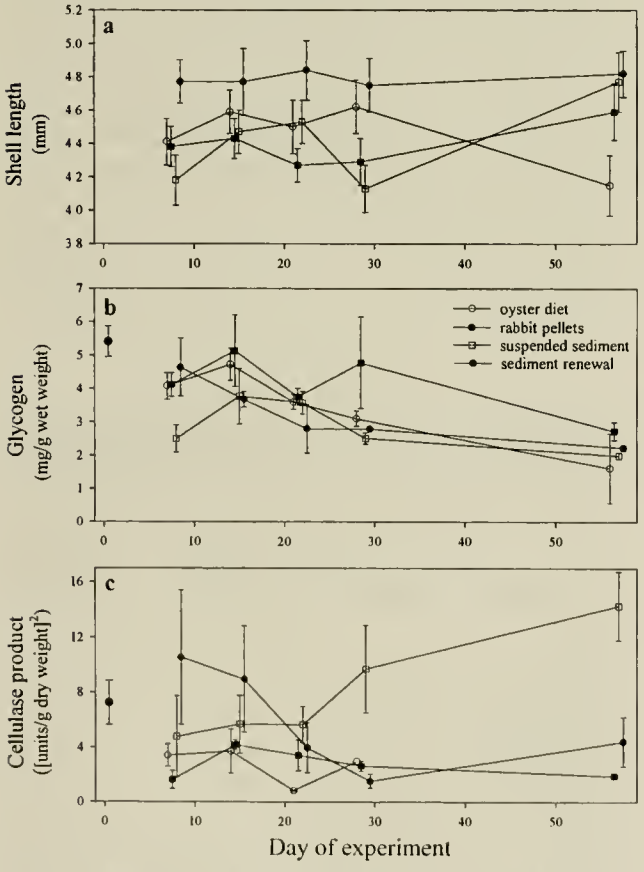

Figure 3. Mean (a) growth, (b) glycogen concentrations, and (c) cellulase activity in fingernail clams *Musculium transversum* fed one of four diets in a 112-day laboratory test. Glycogen (mg/g wet weight) and cellulase activity ([units/g dry weight]²) were measured on a composite of five clams from each of three experimental units sampled on days 7, 14, 21, 28, and 56. Data point at day 0 is the mean (±1 SE) glycogen and cellulase in clams at the time they were collected from the Upper Mississippi River.

In contrast, cellulase activity did not vary among diets (P = 0.12) nor with time held in the laboratory (P = 0.32; Fig. 3c). Cellulase activity, averaged over the 56-day exposure, ranged from 0.8 to 5.3 (units/g dry weight²) in the oyster diet, 0.8 to 4.8 in the rabbit pellets, 1.1 to 14.7 in the suspended sediment, and 0.6 to 19.8 in the sediment renewal. Likewise, cellulase activity remained similar throughout exposure (averaged over all diets) and ranged from 1.6 to 10.5 at day 7 and from 1.8 to 14.3 at day 56. The lack of significant diet or time effects was presumably due to the large variance in cellulase activity among replicates. The coefficient of variation (CV) usually averaged well over 50%, likely obscuring any diet or time effects. For reference, cellulase activity averaged 7.3 ± 1.6 (SE) in clams when collected from the Mississippi River.

DISCUSSION

Survival of fingernail clams was greater in treatments containing sediment from the Upper Mississippi River than in treatments with commercial diets. A similar observation was made by Gatenby et al. (1996) with juvenile *Villosa iris*. In a 45-day laboratory experiment, juvenile mussels reared on sediment and algae had significantly higher survival (67%) than juveniles reared without sediment and fed only algae (22%). Although several investigators have observed higher survival rates in molluscs in experiments with sediment, relative to no sediment (Gatenby et al. 1996, Naimo et al. 2000, present study), the mechanism(s) contributing to this are largely unknown. It has been hypothesized that the addition of a food source, along with fine sediments and their associated resident bacteria, may enhance digestion in molluscs (Crosby et al. 1990, Naimo et al. 2000). However, the addition of bacteria common to riverine systems did not improve survival or enhance growth in laboratory studies with juvenile *Villosa iris* (Gatenby et al. 1996). Naimo et al. (2000) hypothesized that physical contact with sediment may enhance the survival of fingernail clams relative to exposures without direct sediment contact. They observed that *Musculium transversum* were twice as likely to survive when provided with direct sediment contact, suggesting that clams received nutritional benefit from sediment contact by feeding directly on indigenous, sediment-associated food sources.

Although survival of fingernail clams differed substantially among diets after 112 days, survival exceeded 80% through day 21 in all diets. In standardized toxicity tests with benthic invertebrates, 21–28 days is a standard test duration (American Society for Testing and Materials 1992), and tests are generally considered unacceptable if survival of control animals is less than 80%. Thus, in short-term standardized tests with fingernail clams, excessive mortality in control organisms would not invalidate test results.

Growth of fingernail clams in the laboratory was minimal over the 56-day duration. Clams in the sediment renewal treatment seemed to maintain their size, whereas shell growth in clams in the other diets was variable. Differences in shell growth in the sediment-renewal treatment, relative to the other diets, may be related to the volume of available food (i.e., sediment). Clams in the sediment renewal treatment received about 736 g of sediment over 56 days, whereas clams in the suspended-sediment and rabbit-pellet treatments received only 2.1 g of food over this duration. Although food quality as well as quantity are important, the magnitude of the difference in quantity may have contributed to differences in growth among diets. In addition, the magnitude of shell growth observed in our study (0.1–0.6 mm over 56 days) was sufficiently small such that variation in measurement of shell

length could be a major source of variation and uncertainty in this analysis. Thus, future studies should measure individually marked organisms and should use techniques appropriate for detecting small changes in size. The lack of shell growth in this experiment was not unexpected. For example, Gale (1977) observed that *Sphaerium transversum* maintained in the laboratory in chambers containing silt from the Mississippi River grew slowly, with a mean length increase of 1.3 mm after 33 days.

Glycogen concentrations have been used extensively in bivalves as an indicator of physiological health (Haag et al. 1993, Naimo et al. 1998); however, it is unclear how much glycogen is required for maintenance, growth, and reproduction. In the present experiment, we documented significant differences in glycogen concentrations among diets, particularly between the commercial diets and the sediment diets. However, the pattern in glycogen concentrations was such that glycogen was elevated in clams fed the commercial diets, relative to the sediment diets, in contrast to the patterns in survival. Two alternate hypotheses for the reduction in glycogen in the sediment diets include (1) clams were getting enough nourishment from the sediment for maintenance metabolism but were unable to store glycogen and (2) clams were not getting enough nourishment from the sediment and were catabolizing carbohydrate stores. Whichever the case, glycogen concentrations declined with time in all dietary treatments, suggesting that health was declining over this time period. Glycogen concentrations declined by 14–54% by day 7 and 50–70% by day 56, relative to concentrations in clams when they were taken from the river.

Some researchers have suggested that the benefit of addition of sediment to juvenile bivalve cultures is to provide resident bacteria to enhance enzymatic activity (Crosby et al. 1990). However, we did not observe any enhancement in cellulase activity between clams maintained in sediment and clams fed commercial diets. Cellulase activity in clams was highly variable (mean CV = 67%), making detection of dietary effects at an acceptable statistical level difficult. To our knowledge, measurement of cellulase activity has not been previously performed on fingernail clams; thus, further refinement of methods could reduce variation associated with this measure.

In conclusion, we observed significant differences in survival, shell growth, and glycogen concentrations of fingernail clams fed different diets, implying that some diets were better than others. However, the general negative slope of most response variables (survival, shell growth, and glycogen) suggests that clams were declining in health with time in the laboratory, regardless of diet. Therefore, a better diet is needed to maintain clams in a healthy state in the laboratory. Although clams maintained an acceptable survival rate for 21 days in the laboratory, their physiological condition was compromised much earlier. Thus, valid short-term toxicity tests with fingernail clams can be conducted in the laboratory, but their ability to predict toxicity to field populations is uncertain. Therefore, laboratory tests with clams should include a physiological measure, such as glycogen, in addition to survival to ensure that clams are in suitable condition before and during testing in laboratory studies.

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LOSS OF GENETIC VARIATION IN A STRONGLY ISOLATED AZOREAN POPULATION OF THE EDIBLE CLAM, *TAPES DECUSSATUS*

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ABSTRACT We used allozyme electrophoresis to compare the genetic variation of an introduced and strongly isolated population of the edible clam *Tapes decussatus* in the Azores (Lagoa de Santo Cristo, São Jorge) with populations from the main range of the species (Ria and Thau). Observed and expected heterozygosity values, number of polymorphic loci, and mean number of alleles per locus in the main-range populations fall within the limits reported for *T. decussatus* and other Venerid clams. In contrast to previous studies on Venerid clams, we observed no heterozygote deficiencies. In the introduced Lagoa population, we observed a strong reduction of allelic diversity and expected heterozygosities and an effective population size of only 5.30. The Lagoa population is only slightly differentiated from populations from the species' main range and may thus be of low "biological value." Exploitation of *T. decussatus* could therefore be allowed to continue but must follow strict collection guidelines, especially given that only 15% of the area is suitable for exploitation. Otherwise, a unique component of the Azorean fauna that also serves as a fishery resource may be lost.

KEY WORDS: Azores, allozymes, founder effect, *Tapes decussatus*, population genetics, conservation

INTRODUCTION

Small or isolated populations can contribute substantially to biodiversity, and the conservation of such populations must be an important part of any effective Biodiversity Action Plan (Usher 1997). The genetic effects in small populations are manifold (Harris 1984, Usher 1987). Small effective population sizes (N_e) often show a loss of genetic variability (i.e., founder effects, bottlenecks) caused by genetic drift (Lacy 1987). Apart from losing (rare) alleles, small populations often lose common alleles by chance (Nei et al. 1975, Simberloff 1988) and may show elevated inbreeding, which may impair reproductive fitness. In addition, the loss of genetic variability may limit the ability of a population to adapt to changing environments (Frankel and Soulé 1981, Thorpe et al. 1995). Over the long term, these effects may enhance the risk of extinction (Soulé 1987). Effective conservation or management plans require a thorough knowledge of the genetic population structure before adequate measures can be taken. In this study, we estimated the effective population size and investigated whether genetic variation is reduced in an introduced, isolated population of the commercial edible clam *Tapes decussatus* (Linnaeus, 1758).

The main range of *T. decussatus* extends from Great Britain in the north to Senegal in the south, along the Iberian peninsula, and into the Mediterranean to the east (Tebble 1966). Outside its main range, the species has been introduced in the Lagoa de Santo Cristo, a small and isolated lagoon situated at the north coast of the island of São Jorge in the Azores, approximately 1,400 km from the African/European coasts, where it was discovered for the first time in 1967 (Morton 1967). This lagoon has a total area of 0.86 km^2 (length, 500 m; width, 250 m; and maximum depth, 6 m) and harbors a unique fauna (Morton 1967, Santos 1985, Santos and Martins 1986, Morton and Tristão da Cunha 1993, Morton et al. 1998). The lagoon was classified as a Natural Partial Reserve in 1984 on the basis of its unique origin, geology, and the presence of the edible clam *T. decussatus*. In 1989, it was also declared a Special Ecological Area, to safeguard the unique breeding population of *T. decussatus* and to maintain the ecological equilibrium

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of the area. Although there is no written record, *T. decussatus* was probably introduced in the lagoon by humans, especially since the species occurs nowhere else in the Azores (Morton 1967, Morton and Tristão da Cunha 1993). Moreover, the planktonic stage of the larvae lasts approximately 10 days, during which larvae are transported by sea currents over a distance of 10–100 km (Borsa et al. 1991). Larval transport from the main range to the Azores by sea currents seems therefore unlikely.

At this moment, *T. decussatus* is the main commercially exploited species of the lagoon (Fonseca et al. 1995). Santos and Martins (1987), Santos et al. (1989), and Gonçalves and Martins (1991) showed that the population of *T. decussatus* in the Lagoa de Santo Cristo was declining through overexploitation, especially in the intertidal parts of the lagoon, where clam collection is easy. The intense fishery resulted in smaller individuals in the intertidal area. These potential detrimental impacts on the clams and other species of the lagoon have obliged the Azorean government to establish a management program for the Lagoa de Santo Cristo. Therefore, the clam fishery at the lagoon is nowadays closed during a period that largely coincides with the breeding season of the species (May 15 to August 15; Santos and Martins 1987, 1991). The present research was performed to provide genetic data that may be relevant for further substantial management of the clam population.

MATERIALS AND METHODS

Four samples of *T. decussatus* were collected from three sites: Lagoa de Santo Cristo (SC; July 1992 and June 1993), Étang de Thau (Thau; French Mediterranean coast; August 1993), and Playa do Testal (Ria; Ria de Muros y Noya, Galicia, Spain; December 1993). Specimens were immediately frozen in liquid nitrogen for transport to the laboratory, where they were stored at −80 °C.

Forty specimens of each sample were surveyed for allozyme variation with vertical polyacrylamide gel electrophoresis (PAGE). Individual tissue homogenates were prepared by dissecting specimens in ice-cold distilled water and removing the digestive gland, the gills, the foot muscle, and the adductor muscles. Each of the tissues was separately weighted and homogenized in a 20% (w/v) aqueous sucrose solution (5 μL sucrose solution per mg tissue). Crude homogenates were centrifuged for 45 min at ±27,000 *g* at 5 °C to obtain clear supernatants for electrophoresis.

PAGE was performed as described by Backeljau (1987, 1989). Two electrophoretic buffer systems were used: (1) Tris/glycine pH 9.0 in the tray and Tris/HCl pH 9.0 in the gels and (2) Tris/citric acid pH 8.0 in both the tray and the gels. Enzyme staining recipes were adapted from Harris and Hopkinson (1976).

Twenty-six enzyme systems were screened in the four tissues (see Backeljau et al. 1994). Seven of these enzymes yielded interpretable genetic polymorphisms and were retained for further analysis (Table 1).

Alleles were designated alphabetically according to decreasing electrophoretic mobilities (A = most anodal = fastest-migrating allele). Previously typed specimens were included with each run to compare different gels. The BIOSYS-1 version 1.7 package (Swofford and Selander 1981) was used for estimating allele frequencies, mean numbers of alleles per locus (MNA), observed heterozygosities (H_o, direct count) and Nei's (1978) unbiased expected heterozygosities (H_e). Numbers of polymorphic loci (P) were simply counted. Weir and Cockerham's (1984) fixation indices (F_{is}) were estimated with GENEPOP version 3.0 (Raymond and Rousset 1995), and genotype frequencies were evaluated for departures from Hardy-Weinberg (HW) equilibrium expectations with the probability test implemented by the same program. The significance of F_{is} values was tested with FSTAT version 1.2 (Goudet 1995). Linkage disequilibria (LD) between loci were tested with the exact probability test in GENEPOP version 3.0. Whenever needed, testing procedures were corrected for multiple testing with the sequential Bonferroni method (Rice 1989). Nei's (1978) unbiased genetic distance between populations was calculated with BIOSYS-1 version 1.7.

The effective population size (N_e) of the population from the Lagoa was estimated in two different ways. One method estimates N_e from the changes in expected heterozygosity. In a population of size N_e, the initial heterozygosity (H_o) will decrease to H_t after t generations. The relationship between H_o and H_t is given by the equation $H_t = H_o(1 - 1/2N_e)^t$ (Crow and Kimura 1970). A second method (i.e., the temporal method) estimates N_e from temporal changes of gene frequencies as described by Waples (1989) and Hedgecock et al. (1992). Although a few *T. decussatus* individuals may spawn in their first year (Vilela 1950), the vast majority of individuals reach their sexual maturity at the beginning of their second year (Gallois 1977). Therefore, we used a generation time of 1 y for *T. decussatus*. An assumption of both methods is that the allozyme polymorphisms studied are selectively neutral. To test this, we performed the Ewens-Watterson test using the algorithm given in Manly (1985) and implemented by the program POPGENE version 1.31 (updated version of POPGENE version 1.2 of Yeh and Boyle [1997]).

Because many bivalves show a positive correlation between

TABLE 1.

Enzymes studied, E.C. numbers, enzyme codes, the tissue from which the enzyme was extracted, and the buffer system (TC = Tris/citric acid; TG = Tris/glycine) used to examine genetic variation in four *T. decussatus* populations.

Enzyme	EC Number	Code	Tissue	Buffer
Malate dehydrogenase	1.1.1.37	*Mdh*	Adductor muscle	TC
D-Octopine dehydrogenase	1.5.1.11	*Opdh*	Adductor muscle	TC
Isocitrate dehydrogenase ($NADP^+$)	1.1.1.42	*Idhp*	Digestive gland	TC
Phosphogluconate dehydrogenase	1.1.1.44	*Pgdh*	Digestive gland	TC
3-Hydroxybutyrate dehydrogenase	1.1.1.30	*Hbdh*	Digestive gland	TG
Leucylalanine peptidase	3.4.13.11	*Pep*	Gills	TG
Phosphoglucomutase	5.4.2.2	*Pgm*	Adductor muscle	TG

shell size and individual heterozygosity (e.g., Zouros and Foltz 1984), we checked for such a relationship to avoid the possibility that discrepancies in H_o values would merely reflect size differences between populations. Therefore, Pearson's product-moment correlation was calculated between shell length and numbers of heterozygous loci, as outlined by Diehl and Koehn (1985) and Fevolden (1992).

RESULTS

Pep revealed two independent banding zones, the cathodal of which was clearly polymorphic in the Thau and Ria populations, but monomorphic in the Lagoa population. Yet, because the bands in this zone were often confused, they were not used for genotypic analysis. The six remaining enzymes yielded information for seven putative loci (Table 1), the population genetic data of which are provided in Tables 2 and 3. Out of 18 HW tests, only 2 were significant (*Pgm* in Thau and *Idhp* in Ria; Table 2), but this was no longer so after sequential Bonferroni correction. Not surprisingly, F_{is} values taken over all loci in all populations were not significantly different from 0 ($0.193 < P < 0.27$). However, compared with the Lagoa population, the Thau and Ria populations had higher heterozygosity levels and nearly twice as many polymorphic loci and mean numbers of alleles per locus (Table 2). Only two of the 31 LD tests were significant (data not shown), but both cases were no longer significant after sequential Bonferroni correction. Nei's (1978) unbiased genetic distance between the samples ranged from 0.036 (between two samples from the Azores) to 0.23 (between Thau and two samples from the Azores) (Table 3).

The estimate of N_e with the temporal method was infinity. This result is probably an artifact caused by the small number of loci analyzed ($n = 3$) (Table 2). It simply indicates that the change in allozyme frequencies observed between the 2 years was not large enough to be distinguished from sampling error. The estimate of N_e obtained from the reduction of heterozygosity was 5.30. The test for neutrality gave nonsignificant results.

We found no significant correlation between individual heterozygosity and shell length (Thau, $r = 0.173$, $P = 0.733$; Ria, $r = 0.36$, $P = 0.556$; and Lagoa (pooled samples), $r = 0.48$, $P = 0.409$).

DISCUSSION

Observed and expected heterozygosity values, number of polymorphic loci, and mean number of alleles per locus in the Ria and Thau populations fall within the limits reported for *T. decussatus* and the palourde *Ruditapes philippinarum* (Table 4). As in many other bivalve species, heterozygote deficiencies have often been reported in *T. decussatus* and *R. philippinarum* (see references in Table 4), but at present the causes of this remain unclear (Zouros et al. 1988). Yet, in our study, we observed no heterozygote deficiencies. Nevertheless, our population genetic data of the Thau population are very similar to the results obtained by Jarne et al. (1988), Borsa and Thiriot-Quiévreux (1990), and Borsa et al. (1994) for the same population and for the nearby population of Étang du Prévost (Worms and Pasteur 1982). Moreover, genetic distances between our populations are similar to those reported by Jarne et al. (1988) (compare our Table 3 with their Table 4).

However, in the Lagoa population of *T. decussatus* in the Azores, we observed a strong reduction of allelic diversity and expected heterozygosities, but not heterozygote deficiencies, compared with main-range populations. Substantial losses of genetic diversity have also been observed in bivalves for which hatchery stocks have been established from only a few individuals (e.g., the oysters *Crassostrea gigas* [Gosling 1982, Hedgecock and Sly 1990] and *C. virginica* [Vrijenhoek et al. 1990, Gaffney et al. 1992]). This may have important implications when management and exploitation practices are developed. Many hatchery stocks or introduced populations have a low N_e value despite densities that can be very high (e.g., Saavedra 1997 and references therein). In the Lagoa, population densities of *T. decussatus* may reach 400 individuals/m^2 (Gonçalves and Martins 1991). Yet we estimated an effective population size of only 5.30 individuals. Founder effects, genetic drift, intentional selection, and inadvertent selection during culture are likely to reduce the genetic diversity of the Lagoa population further. The introduction of a small number of individuals a few decades ago probably resulted in the loss of genetic variation via founder effects. The strong isolation of this population probably does not allow transport of larvae from nearby populations (see Introduction), and genetic drift and inbreeding may further reduce genetic variability. These effects are probably reinforced by human activities such as selection during harvesting (e.g., the collection of only large adults). Indeed, the exploitation of *T. decussatus* in the Lagoa follows a classic "fishery" picture with old (i.e., large) shells lacking among empty shells in the lagoon because they were collected for consumption when alive (Morton and Tristão da Cunha 1993). It is unclear whether such selective harvesting affects the genetic structure of the population, because there was no association between individual heterozygosity and size. Yet this topic deserves further study, as Borsa et al. (1994) and Passamonti et al. (1997) found a high level of intrapopulation structuring, probably related to year-cohort heterogeneities, that perhaps indicate short-term selection or genetic drift (Borsa et al. 1994). Thus, harvesting a single age cohort (i.e., oldest and largest individuals) could affect the genetic population structure.

In none of the populations did we observe a significant correlation between shell size and individual heterozygosity. Some other studies also failed to show a relationship between individual heterozygosity and morphological traits such as size and growth (Adamkewicz et al. 1984, Volckaert and Zouros 1989, Gaffney 1990, Slattery et al. 1991), but others report negative (Wilkins 1978) or positive (Garton et al. 1984, Koehn and Gaffney 1984, Zouros and Foltz 1984, Gaffney 1990) associations, although associations may differ among populations (Gaffney 1990).

A positive relation between heterozygosity, body size, and survival was found in a population of *T. decussatus* that survived natural anoxic stress (Borsa et al. 1992). However, in other populations of the same species, Jarne et al. (1988) observed no association between asymmetry of left and right valves (as a measure of fitness, i.e., the more asymmetric the less fit) and heterozygosity, and an increased variance for morphological traits in the classes with low heterozygosity. This also appears to be the case for some of the *R. philippinarum* populations in the Po river lagoon in Italy (Fava et al. 1994). In that study, individual heterozygosity and phenotypic variability appeared to be negatively correlated, but the relationship was heterogeneous between populations (Fava et al. 1994).

TABLE 2.

Allozyme variation in four populations of *T. decussatus* (for full population names we refer to the text).

	Thau (n = 40)	Ria (n = 40)	SC92 (n = 40)	SC93 (n = 40)
Mdh				
A	0.837	1.000	1.000	1.000
B	0.163			
H_e	0.272			
H_o	0.325			
F_{is}	−0.182			
P_{exact}	0.564			
Opdh				
A	0.625	0.538	0.488	0.600
B	0.213	0.225	0.262	0.212
C	0.162	0.237	0.250	0.188
H_e	0.538	0.604	0.631	0.560
H_o	0.575	0.575	0.675	0.575
F_{is}	−0.057	0.061	−0.057	−0.015
P_{exact}	0.500	0.801	0.526	0.458
Idhp				
A	0.113	0.038		
B	0.887	0.962	1.000	1.000
H_e	0.200	0.072		
H_o	0.125	0.025		
F_{is}	0.385	0.661		
P_{exact}	0.057	0.038*		
Pgdh				
A	0.138			
B	0.200	0.225	0.462	0.375
C	0.349			
D	0.175	0.613	0.338	0.400
E	0.138	0.162	0.200	0.225
H_e	0.769	0.548	0.632	0.649
H_o	0.700	0.525	0.650	0.650
F_{is}	0.102	0.054	−0.016	0.011
P_{exact}	0.384	0.881	0.973	0.378
Hbdh-1				
A	0.250	0.225		
B	0.724	0.762	1.000	1.000
C	0.013	0.013		
D	0.013	0.013		
H_e	0.412	0.368		
H_o	0.400	0.275		
F_{is}	0.041	0.264		
P_{exact}	0.832	0.144		
Hbdh-2				
A	0.987	1.000	1.000	1.000
B	0.013			
H_e	0.025			
H_o	0.025			
F_{is}	−0.013			
P_{exact}	1.000			
Pgm				
A	0.400	0.586	0.887	0.937
B	0.537	0.363	0.113	0.063
C	0.063	0.038		
D		0.013		
H_e	0.547	0.522	0.200	0.117
H_o	0.675	0.500	0.175	0.125
F_{is}	−0.222	0.055	0.136	−0.054
P_{exact}	0.011*	0.192	0.396	1.000

TABLE 2.

Continued

	Thau (n = 40)	Ria (n = 40)	SC92 (n = 40)	SC93 (n = 40)
Overall				
H_e	0.400	0.306	0.212	0.192
(SE)	(0.096)	(0.104)	(0.114)	(0.110)
H_o	0.404	0.271	0.214	0.193
(SE)	(0.100)	(0.100)	(0.118)	(0.110)
MNA	3.0	2.4	1.7	1.7
P	7/7	5/7	3/7	3/7
$P_{(+Pep)}$	8/8	6/8	3/8	3/8

H_e, expected heterozygosity; H_o, observed heterozygosity; F_{is}, fixation index; P_{exact}, exact P-values (*$P < 0.05$); MNA, mean number of alleles per locus; P, proportion of polymorphic loci; SE, standard error.

Our allozyme data indicate that the Lagoa population from the Azores is genetically depauperate and only slightly differentiated from populations from the main range and may thus be of low "biological value" (i.e., in terms of biodiversity). Gathering of *T. decussatus* could therefore be allowed to continue. Nevertheless, given the lower genetic diversity of *T. decussatus* in the Lagoa, the low effective population size, and the depauperate intertidal region (Santos et al. 1985, Santos and Martins 1987), exploitation of this species must follow strict collection guidelines (see also Santos 1989), especially given that only 15% of the area is suitable for exploitation (Morton and Tristão de Cunha 1993). Otherwise, a unique component of the Azorean fauna that also serves as a small fishery resource may be lost. In addition, there is much to compare between Ilhéu de Vila Franca on the island of São Miguel in the Azores and the Lagoa de Santo Cristo. The faunistic and scientific value of Ilhéu de Vila Franca is strongly reduced because of tourism. Thus, opening up the Lagoa for tourism could be disastrous for the fauna too. Therefore, in view of the unique origin, geology, fauna, and flora, the place should be declared a "Site of Special Scientific Interest" (Morton and Tristão da Cunha 1993).

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TABLE 3.

Nei's (1978) unbiased genetic distance between the four populations of *T. decussatus* (for population names we refer to the text).

	Thau	Ria	SC92	SC93
Thau				
Ria	0.152			
SC92	0.239	0.129		
SC93	0.230	0.129	0.036	

TABLE 4.

Allozyme variation reported in other studies of *T. decussatus* and *R. philippinarum*.

Species	H_o	H_e	MNA	P	Reference
T. decussatus					
		0.28	2.75	0.83	Worms and Pasteur (1982)
		0.23–0.28	2.18–2.73	0.64–0.73	Jarne et al. (1988)
	0.22	0.26	2.33	0.78	Borsa and Thiriot-Quiévreux (1990)
	0.18–0.24	0.23–0.33	1.54–1.99	0.54–0.66	Passamonti et al. (1997)
	0.19–0.40	0.19–0.40	1.71–3.00	0.43–1.00	This study (all populations)
R. philippinarum					
	0.26	0.26	3.18	0.73	Moraga (1986)
	0.16–0.20	0.18–0.22	2.67–3.44	0.22–0.33	Kijima et al. (1987)
	0.17–0.25	0.20–0.27	2.6–3.6	0.43–0.57	Oniwa et al. (1988)
	0.33	0.34	2.89	0.89	Borsa and Thiriot-Quiévreux (1990)
	0.34–0.37		2.80–3.10	0.80–0.93	Fava et al. (1994)
	0.19–0.22	0.20–0.27	1.57–1.63	0.54–0.75	Passamonti et al. (1997)
	0.27	0.27	3.15–3.35	0.75–0.85	Yokogawa (1998)

H_o, observed heterozygosity; H_e, expected heterozygosity; MNA, mean number of alleles per locus; P, percentage of polymorphic loci.

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LIFE HISTORY AND HABITAT OBSERVATIONS OF SOFTSHELL CLAMS *MYA ARENARIA* IN NORTHEASTERN NEW JERSEY

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ABSTRACT Population densities, survival, factors associated with mortalities, and growth of softshell clams, *Mya arenaria*, in two northeastern New Jersey estuaries were studied from 1993 through 1997. The study areas were near shore where low-tide water depths ranged from 15 to 90 cm. Juvenile densities were high only in 1993. Light sets of juveniles from 1994 to 1997 disappeared by the end of their first summer. The longest living softshells were the abundant 1993 year class, which survived for 26 mo in the Shrewsbury River. This contrasts with life spans of 7–12 years for softshells in New England. Mortalities of softshells were correlated with: (1) predation by the striped killifish, *Fundulus majalis*, and mummichog, *F. heteroclitus;* (2) mats of sea lettuce, *Ulva lactusa*; and (3) high temperatures (30–32 °C). Softshell sarcoma was also present and may have contributed to mortalities. The effects of the mortality agents varied among locations and years. The softshells of the Shrewsbury River averaged about 23 mm and 40 mm long at the end of their first and second growing seasons, respectively.

KEY WORDS: *Mya arenaria*, settlement densities, survival, mortality factors, growth

INTRODUCTION

The softshell clam, *Mya arenaria*, ranges along the Atlantic coast of North America from Labrador (Abbott 1974) to Georgia (Rasmussen and Heard 1995), with the highest abundances located from the Bay of Fundy to Chesapeake Bay. The species also occurs in Europe and has been successfully introduced to the coasts of western North America (Abbott 1974). Investigators in New England commented on the wide variations in magnitude of annual sets and on the subsequent survival of softshells (Belding 1930, Turner 1949, Turner 1950, Brousseau 1978b). Softshells can live as long as 7 y (Brousseau 1978b) to 12 y (Belding 1930). Most postsetting mortalities of softshells are caused by predation by shrimp, fish, ducks, brachyuran crabs, xiphosuran crabs, and naticid snails (Belding 1930, Turner 1949, 1950, Foley and Taber 1952, Glude 1955, Smith et al. 1955, Cronin and Hall 1968, Palmer 1976, Edwards and Huebner 1977, Kelso 1979, Holland et al. 1980, Commito 1982, Hines et al. 1990, Eggleston et al. 1992, Rasmussen and Heard 1995), and by breakage and displacement in storm-exposed areas (Kellogg 1910, Belding 1930, Turner 1950, MacKenzie and Stehlik 1988). Investigators in Europe also have reported on the wide annual variability in densities of softshell juveniles and on their subsequent survival and causes of mortality (DeVlas 1979, Beukema 1982, Pihl 1982, Moller and Rosenberg 1983, Kube 1996).

Epizootics of softshells reported from New England to Chesapeake Bay have been associated with disseminated sarcomas (Barry and Yevich 1972, Farley 1976, Yevich and Barszcz 1977, Brown et al. 1977, 1979, Farley et al. 1986, Brousseau 1987, Barber 1990). The proliferative condition is transmissible, progressive, and usually fatal (Brown 1980, Cooper et al. 1982, Farley et al. 1986). The etiology of softshell sarcoma is uncertain; environmental factors (Barry and Yevich 1972, Yevich and Barszcz 1977) and a viral agent (Oprandy and Chang 1981) have been suspected.

The Navesink and Shrewsbury Rivers and nearby Raritan Bay in northeastern New Jersey have produced softshells since prehistoric times (MacKenzie 1990, MacKenzie 1992), but in recent years the stocks have been small, and, consequently, commercial production usually has been small or nonexistent. Previous studies of the softshells in this area have described abundances (Dean 1975), longevity (Appeldoorn 1983, Appeldoorn 1995), abundances and effects of storms (MacKenzie and Stehlik 1988), and the incidence of sarcoma (Barber 1990). Our study was undertaken to characterize annual recruitment, survival, factors that cause mortality, and growth.

Study Areas

The study areas were in the Navesink and Shrewsbury Rivers, in New Jersey (Fig. 1). The primary study site in the Navesink River was off its southeast shore. The site comprised about 3 acres of firm muddy-sand bottom and extended from near the shore edge to about 75 m offshore; water depths were from 15–90 cm at low tide. The mean tidal amplitude is about 1.7 m (Jeffries 1962). Mats of sea lettuce, *Ulva lactuca*, formed in the site, and their aerial distributions varied widely among years. The study site in the Shrewsbury River was off its northeast shore at a similar shore position and water depth, and its bottom sediments were similar. It was about 1 acre in size. Little sea lettuce grows in that section of the river. A reason for selecting the two sites was convenient access to the shore by foot as most all the shoreline areas of the two rivers are private property. The softshells in the two rivers are subtidal.

The identified predators of softshells in the two rivers were: the striped killifish, *Fundulus majalis;* the mummichog, *Fundulus heteroclitus;* and the blue crab, *Callinectes sapidus*. Schools of striped killifish and mummichogs were nearly always present in the study sites, except during the lowest tides, from at least mid-May into October. The blue crabs were scarce in the rivers from 1993 to 1996, but were more abundant in 1997.

During this study, the salinity at the Navesink River site ranged from 15 to 25 0/00, and at the Shrewsbury River site from 20 to 25 0/00. Water temperatures were mostly 11–12 °C during early May, 18–20 °C during June, and peaked at about 25 °C in late July and early August, but in mid-afternoon during late July-early August, 1995, water temperatures ranged from 30.0 °–31.8 °C. Temperatures afterward cooled.

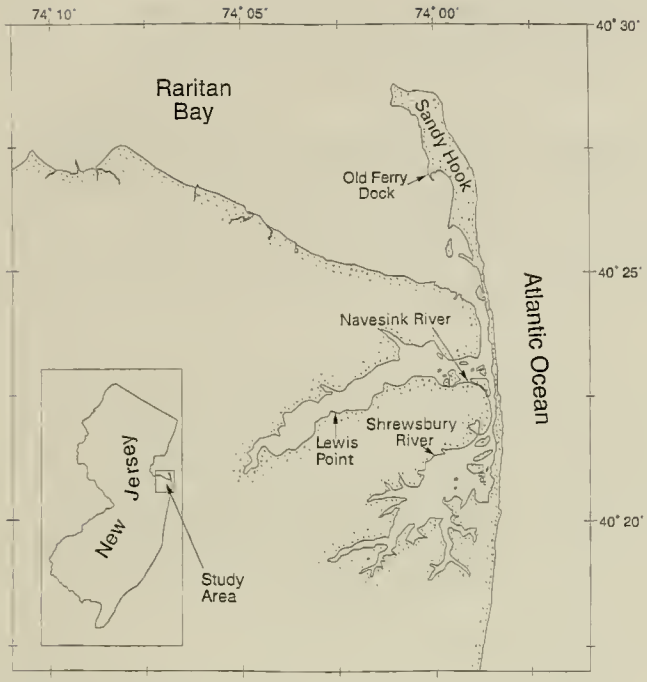

Figure 1. Locations of study and sampling sites in northeastern New Jersey.

The waters of the Navesink and Shrewsbury Rivers interchange with Raritan Bay, which is contaminated with many types of pollutants (Pearce 1983, Breteler 1984). The pollutants consist of suspended particulates, oil and grease, many toxic trace metals, polynuclear aromatic hydrocarbons, polychlorinated biphenyls, DDT, and dioxins (Stanford and Young 1988, Wolfe et al. 1996). In 1974, the copper concentration in western Raritan Bay bottom water was 65 parts per billion (ppb), the highest reported for any estuary; the copper concentration in the surface water there was 36 ppb, and in mid-Raritan Bay it was 7.9 ppb (Waldhauer et al. 1978). In 1992, the copper concentration in the surface water was considerably lower: 4.6 ppb in western Raritan Bay, and 4.3 ppb in mid-Raritan Bay (Anonymous 1992). The bay has extremely high primary productivity with the annual value in the 1970s at 817 g C/m^2, which was considered among the highest of any estuary (O'Reilly et al. 1976). In the 1960s, Raritan Bay was classified as an advanced eutrophic system (Federal Water Pollution Control Administration 1967), but since the 1970s its water quality has improved (Brosnan and O'Shea 1995). Elevated nitrogenous wastes nevertheless continue to stimulate the growth of dense phytoplankton blooms (Draxler et al. 1984, Brosnan and O'Shea 1995); Draxler et al. (1984) had reported Secchi disc readings in the bay of < 1.0–2.0 m during most of the spring and summer. Steimle and Caracciolo-Ward (1989) have shown that the density and diversity of benthic macrofauna in Raritan Bay are relatively low compared with other U.S. east coast estuaries. Similar determinations of pollution, phytoplankton blooms, and macrofauna have not been reported in the Navesink and Shrewsbury Rivers.

The eutrophication of waters probably was responsible for producing some large mats of sea lettuce observed in the two rivers. As Hull (1987) noted, sea lettuce begins as tiny leaves attached to shells and other objects in the spring, grows and persists as thick mats during the summer, and then nearly disappears in the late fall.

METHODS

Sampling Procedures

Field observations lasted 5 y, 1993 to 1997. Water salinity was determined quarterly by titration. Surface water temperatures were measured with a hand-held thermometer daily at 7:30 AM at the Navesink River study site from May into September in 1994, 1995, and 1996. In 1993, following heavy sets of softshells, densities of this year class were estimated once a month, except in the coldest parts of the year, in the Navesink and Shrewsbury River study sites, by placing a ring that encircled a 0.28 m^2 area on the substrate and then removing all softshells for counting and measuring. Three such samples were taken for each determination. From each monthly sample, a subset of 100 softshells, was measured and lengths were plotted to determine growth rates. In 1994, samples to determine the densities of young-of-the-year (juvenile) softshells were taken similarly at each site. In 1995, 1996, and 1997, three 0.28-m^2 areas or six 0.1-m^2 areas were sampled at each site. Only two to three samplings were made in each year from 1994 to 1997, because the low densities of juvenile softshells fell quickly to nearly zero per sample following the initial samplings in June or July.

Potential predators of softshells were collected by pulling a fine-mesh, 15-m seine for about 60 m over an inshore section of bottom in the study sites in the Navesink and Shrewsbury Rivers. A single seining was made at each site at half tide during the outgoing tide in July 1994. Fish and shrimp were collected, but only the fish were examined. They were placed in a plastic bag, held on ice in a cooler, and frozen the same day. Later, they were thawed, and the invertebrates, plants, and other contents in their stomachs and guts were identified and counted using a dissecting microscope.

Field Experiment on Fish Gut Evacuation

During August 1996, an experiment was conducted to determine the evacuation rate of food from the stomachs and guts of the mummichog, *F. heteroclitus*. One hundred mummichogs (mean length 79.1 mm; range 63–110 mm) were seined and divided into five groups of 20 each. The first group of fish was immediately iced, then frozen, and later thawed and examined for the quantity of food in their guts. The other four groups were held in separate field cages suspended above the bottom for 3, 6, 9, and 24 h at temperatures of 23.5 °–25.0 °C and then processed similarly to the first group. A visual estimate was made of gut fullness.

Diagnosis of Sarcomas

The prevalences of softshell sarcomas were determined using histological methods (Farley et al. 1986). Samples of 50 softshells, 40–55 mm long, were collected quarterly at four sites, namely, our two primary study sites in the Navesink and Shrewsbury Rivers, at Lewis Point (5 km west of our primary study site in the Navesink River), and in Raritan Bay at the Old Ferry Dock on the west side of Sandy Hook (Fig. 1). The collections eventually ended in the Navesink and Shrewsbury Rivers because the softshells had died or had became too scarce. Following collections, the softshells were transported to the Cooperative Oxford Laboratory, Oxford, MD. Hemolymph was drawn from the adductor muscles into sterile syringes containing ambient sterile seawater, expelled into slide chambers, and fixed after 30 min in 1 glutaraldehyde-4 formalde-

hyde. The hemolymph preparations were stained with fuelgen picromethyl and were examined for sarcomas by light microscopy.

RESULTS

Navesink River

The setting densities of juvenile softshells in our Navesink and Shrewsbury River study sites were similar to one another each year. The juveniles were relatively abundant in the two rivers only in 1993. In the Navesink River, they had set throughout the shallows over a distance of 10.5 km off its south and northwest shores. At the study site, their density at the initial sampling in August 1993 was 1,110/0.28 m^2. Their survival after that was fairly high: 60–69% were alive in late April to late May 1994 (Table 1).

In 1993, sea lettuce was relatively sparse in the study site, but by mid-June to early July 1994, a solid mat of sea lettuce had formed. The mat was about 25 cm thick and extended from the shore outward to cover about half of the 3-acre bed. In addition, some isolated stationary sea lettuce mats, as small as 2 m across, formed in areas beyond the main mat. All the observed 1993 year class of softshells covered by the mats initially extended their siphons several centimeters out of the sediment, then emerged from it, laid on its surface beneath the mat, and died. In contrast, the softshells in unvegetated areas did not extend their siphons, emerge, and die.

From 1994 through 1997, the sets of juvenile softshells were light in the river. In 1994, the unvegetated sediments outside any sea lettuce mats received a set of juveniles; on June 30 of that year, they had a mean density of 54.7/0.28 m^2 (three replicates, SE 9), but by July 8, 1994, their density had fallen to 2.3/0.28 m^2 (three replicates, SE 0.7). The 1995 and 1996 sets were much more sparse than those in 1994 and 1997. On July 28, 1997, the 1997 juveniles had a mean density of 28.8/0.10 m^2 (six replicates, SE 4.7), but by August 9, 1997, their density had fallen to 3.7/0.10 m^2 (six replicates, SE 0.6). Subsequent samplings in August and September each year from 1994 to 1997 found few juveniles in the site.

On July 7, 1994, when the density of the 1994 year class of softshells was declining rapidly, a seining was made over the bed to examine the stomachs and guts of fish. Forty-one of 60 striped killifish (average length 64 mm, range 46–78 mm) contained an average of 46 juvenile softshells/fish (range 1–169 softshells), and one of three mummichogs (average length 97.3 mm, range 84–115 mm) contained two juvenile softshells. The softshells ranged from 2–11 mm long. The remaining striped killifish and mummichogs had food in their stomachs but no softshells.

TABLE 1.

Densities, mean, and standard error (S.E.) of 1993 year class *Mya arenaria* at study sites in Navesink River and Shrewsbury River. Densities are expressed as mean per 0.28 m^2. S.E. is based on 3 samples on each date.

Navesink River			Shrewsbury River		
Date	Mean	S.E.	Date	Mean	S.E.
1 Sep 93	1,110	117	7 Oct 93	849	57
8 Oct 93	1,170	200	11 Nov 93	650	62
28 Apr 94	668	37	29 Apr 94	677	45
24 May 94	767	16	28 Jun 94	784	85
29 Jun 94	0		2 Aug 94	586	10
			2 Sep 94	520	81
			26 Apr 95	573	9
			7 Jun 95	456	16
			7 Aug 95	0	

Shrewsbury River

In 1993, softshells set densely in the shallows along most of the north shore of the Shrewsbury River in a band about 7 m wide, over a distance of about 4.2 km. The density of the 1993 year class of softshells at the study site at the initial sampling in October 1993 was 849/0.28 m^2. After that, their survival was fairly high, as 54–67% were alive in late April-early June 1995 (Table 1). By August 7, 1995, about 26 months after setting, this entire year class of softshells was dead at the site. They died during a period of unusually high air and water temperatures in late July-early August. At 3:00 PM on July 31, the water temperature was 31.8 °C, the softshells were dying and rotting, and the water over the bed was a yellow-brown mixture of rotting softshell meats and brown phytoplankton. Their mortality apparently was caused by the high temperatures, because the lethal temperature of adult softshells is in the temperature range of 30.5 °–32.5 °C (Kennedy and Mihursky 1971).

From 1994 through 1997, juvenile softshells were relatively scarce throughout the river. At the study site, the small numbers observed by scraping with a sieve through the surface of sediments in 10 places in June and July disappeared by August or September in the years in which they set, similarly as the light sets had disappeared in the Navesink River.

On July 8, 1994, fish were seined at the study site and their guts were examined for softshells and other foods. Four striped killifish (average length 107 mm, range 92–113 mm) contained an average of 26 juvenile softshells/fish (range 21–32 softshells per fish); 123 of 150 mummichogs (average length 69 mm, range 40–93 mm) had an average of 15.5 juvenile softshells per fish (range 1–53 softshells per fish); and one spot, *Leiostomus xanthurus*, had 115 juvenile softshells. The softshells ranged from 4 to 11 mm in length for all fish. Other items in the guts of striped killifish and mummichogs in the Navesink and Shrewsbury Rivers were: juvenile common Atlantic slippersnails, *Crepidula fornicata;* amphipods; isopods; juvenile horseshoe crabs, *Limulus polyphemus* (about 3 mm carapace width); polychaetes; sea lettuce; and detritus.

Food Passage Through Mummichogs

Mummichogs passed food through their stomachs and guts rapidly (Fig. 2). In the experiment to estimate the rate, a large decline (80%) in fullness of their guts was evident after 3 h, and little food remained after 24 h. The results suggest that the softshells found in mummichogs that were seined at the sites were eaten within 24 h, and they imply a high consumption rate.

Histology

In the Navesink River, quarterly samples showed a low sarcoma prevalence in 1994, but prevalence reached 18% in December 1995 and decreased slightly to 13% and 14% for the first two quarters in 1996, while samples from Lewis Point were negative for sarcomas in 1991 to 1993 (Table 2). In the Shrewsbury River, quarterly samples of softshells examined for sarcomas were negative in 1994 and 1995. At the Old Ferry Dock, in collections in

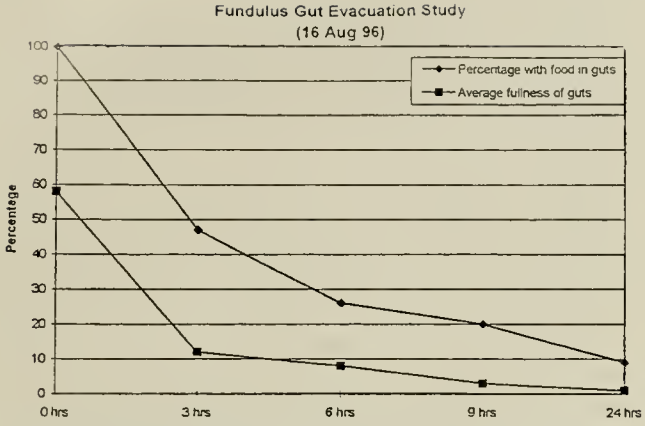

Figure 2. Percentage with food in guts and average fullness of guts of *F. heteroelitus* held in field cages at spaced intervals, 0–24 h.

1995, 1996, and 1997, from 10–20% of softshells were infected with sarcoma on four of seven dates, and from 0–4% were infected in the remaining three dates.

Growth

The length-frequency curves for the 1993 year class of softshells in the Navesink and Shrewsbury Rivers are presented in Figure 3. The curves for each time period show a single mode that broadens somewhat as time passes. In the Navesink River, the softshells had a mean length of 15.4 mm in September 1993, 22.1 mm in November 1993, and 31.6 mm in May 1994. In the Shrewsbury River, their mean lengths were 19.3 mm in October 1993, 22.9 mm in November 1993, 26.6 mm in April 1994, 38.9 mm in November 1994, 47.6 mm in April 1995, and 48.9 mm in June 1995.

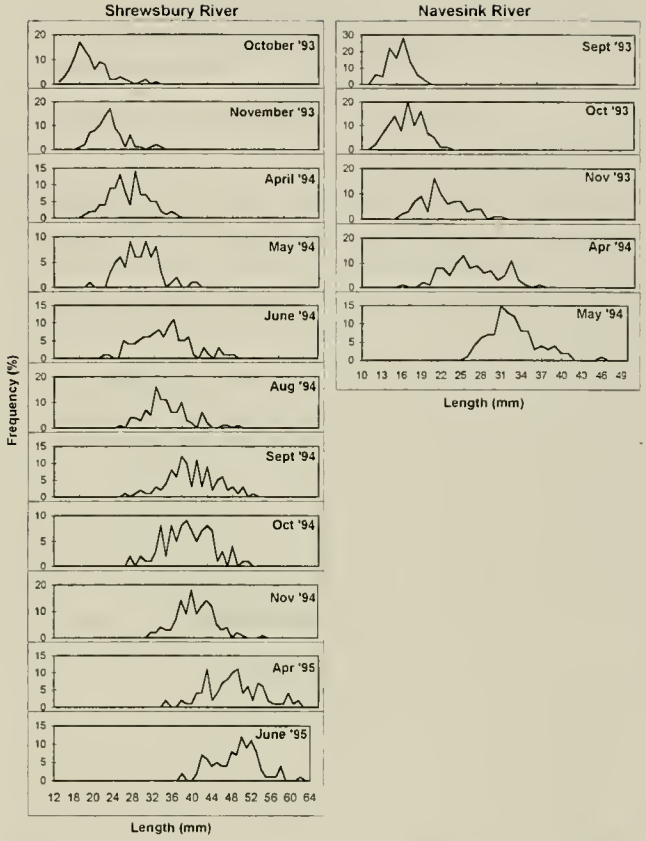

Figure. 3. Length-frequency distributions of the 1993 year class *Mya arenaria* in the Navesink River, 1993 to 1994, and the Shrewsbury River, 1993 to 1995.

TABLE 2.

Percent prevalences of softshell sarcomas based on histology (n = 50).

Date	Lewis Point[a]	Navesink River	Shrewsbury River	Old Ferry Dock
7-9-91	0			
9-4-91	0			
12-4-91	0			
3-3-92	0			
6-3-92	0			
9-9-92	0			
12-9-92	0			
3-25-93	0			
6-38-93	0			
9-29-93	0			
6-15-94		0	0	
9-12-94		4	0	
12-6-94		2	0	
5-22-95		0	0	
6-26-95		2	0	
7-25-95		6	0	
9-21-95		2		4
12-5-95		18		12
3-27-96		13.3[b]		10
7-18-96		14.4[c]		0
10-3-96				10
2-26-96				20
4-14-97				2

[a] Location in Navesink River.
[b] n = 47.
[c] n = 45.

DISCUSSION

In attempting to find reasons for the large annual variability in setting densities of softshells in Europe, Beukema (1982, 1992), Jensen and Jensen (1985), and Moller (1986) observed that heavy sets of softshells and some other bivalves occurred during summers following cold winters and that light sets followed mild winters. The bivalves were active during the mild winters when little food was available in the water, and they consequently had absorbed most of their gonads by the time spawning began in the spring. Our study was not continued sufficiently long enough to document such a correlation, but it is likely that dense sets of softshell juveniles result from certain weather conditions. The spring and early summer of 1993 when the heavy sets occurred in the Navesink and Shrewsbury Rivers did feature weather with no cold easterly winds with rain. During the springs and summers of 1994 to 1997, however, when light sets occurred, several periods of cold easterly winds and rain, each of 3–4 days duration, were interspersed with periods of warmer westerly and southerly winds. Belding (1930) had noted that the numbers of larvae in the water declined during periods of cold rains.

Earlier investigators have noted the disappearances of softshell

juveniles by the end of their first summer in some years (Brousseau 1978a, Moller and Rosenberg 1983, Beukema 1979, Pihl 1982). We believe that predation by striped killifish and mummichogs was the principal reason for the sharp declines and disappearances of juveniles in our study sites during 1994 to 1997. The observations suggest that any relatively light sets of softshells, as dense as 500/m^2 or even higher, could be lost to such predation every year whenever the fish are abundant in the two rivers. The fish likely were present and preyed on juvenile softshells in 1993, but perhaps the juveniles were so abundant that a great many remained alive by the time they had grown too large for the fish to prey on them.

Fish also prey on softshells in other regions. Kelso (1979) described heavy predation of juvenile softshells by mummichogs in Massachusetts. In our study, the sizes of softshells (2–11 mm long) taken by the striped killifish and mummichogs were similar to those that Kelso (1979) reported; probably 11 mm is near the maximum size of a softshell that the fish can devour. More softshells were present in the guts of mummichogs in the Navesink River (about 46 softshells per fish) than he found in Massachusetts (6–9 softshells per fish). Perhaps the softshells were more abundant in the Navesink River. Medcof and McPhail (1952) stated that adult winter flounders, *Pleuronectes americanus*, about 28 cm long, consumed whole juvenile softshells and nipped off the siphon tips of adult softshells in eastern Canada. In their study, the softshells with nipped siphons recovered without unusual mortality. Rasmussen and Heard (1995) stated that Atlantic stingrays, *Dasyatis sabina*, feed on softshells in Georgia. Pihl (1982) and Moller and Rosenberg (1983) observed that flounders *Platichthys flesus* consume large numbers of juvenile softshells, 2–12 mm long, in Sweden, and DeVlas (1979) observed that flounders *P. flesus* and plaice, *Pleuronectes platessa*, consume juvenile softshells and the siphon tips of older softshells in the Netherlands. Summer flounders, *Paralichthys dentatus*, and other fish were present in the Navesink and Shrewsbury Rivers and might have preyed on softshells, but they were not observed or collected during our visits to the study areas.

Relatively scarce in our study sites from 1993 to 1996, blue crabs appeared to be a minor predator then, but they were abundant and may have killed many juvenile softshells in 1997. Since our observations were limited to periods of low and mid tides and during daylight, blue crabs and other predators may have entered the study sites and eaten some juveniles during high tides and at night during all years. Green crabs, *Carcinus maenas*, and naticid snails, both predators of softshells in New England (Belding 1930, Glude 1955, Smith et al. 1955, Edwards and Huebner 1977, Commito 1982), were not observed in the two rivers during 1993 to 1997 and could not have caused much mortality of the softshells. Horseshoe crabs, also a softshell predator in New England (Turner 1949, 1950), were scarce and apparently killed few softshells in the two rivers. The shrimp, *Crangon crangon*, preys on softshells as large as 3 mm long in Europe (Moller and Rosenberg 1983). The seven-spine bay shrimp, *Crangon septemspinosus*, and the marsh grass shrimp, *Palaemonetes vulgaris*, were abundant in our two study areas but were not examined as predators of small postset softshells, and neither were amphipods and isopods.

In eastern North America, greater scaup, *Aythya marila*, prey on a variety of small clams, including softshells, blue mussels, *Mytilus edulis*, and snails (Foley and Taber 1952, Cronin and Hall 1968, Barclay pers. commun., 1998). Black ducks, *Anas rubripes*, prey on bivalves, including *Macoma balthica*, blue mussels, and marine snails, such as eastern mud snails, *Ilyanassa obsoleta* (Palmer 1976). Greater scaup and black ducks were present in the Navesink and Shrewsbury Rivers, but there were no signs that they ate softshells in our study areas.

Juvenile softshells also can be killed on exposed shallow habitats during wind storms by having their thin shells ground into fragments or being washed onto nearby beaches (Kellogg 1910, Belding 1930, Turner 1950, MacKenzie and Stehlik 1988). This type of mortality was not observed in our Navesink and Shrewsbury River study sites, but it was observed in the softshells that had set along the south shore of Raritan Bay.

Once past their first summer, softshells can survive fairly well as long as exogenous mortality factors are absent, as shown by Belding (1930) and Brousseau (1978b) in New England, Kube (1996) in Europe, and others. In the Navesink River, the 1993 year class of softshells survived well from September 1993 through May 1994 until mats of sea lettuce killed them, and in the Shrewsbury River it survived well from October 1993 through June 1995 when shortly afterward high temperatures apparently killed them. The age of the Shrewsbury River softshells when they died, 26 mo, was the maximum that any lived in the two study sites and is far shorter than softshells lived in New England where their habitat was undoubtedly much better (Belding 1930, Hanks 1963, Brousseau 1978b). Appeldoorn (1995) stated that softshells in the Navesink River could live at least 15 years around the time of his sampling (1977), but his finding was based on shell markings and sizes of softshells found during a single collection and might be in error. Nevertheless, in an earlier paper, Appeldoorn (1983), reporting on the same 1977 samples, stated that softshells were present as large as 78 mm long or even larger and were obviously older than the largest softshells (62 mm) that we found in the Navesink and Shrewsbury Rivers. The environmental conditions in the two rivers during 1993-1997, such as extremely high temperatures in 1995, apparently did not allow the softshells to live as long as they did during the 1970s.

Some earlier workers had shown that algal mats grow over and kill bivalves, but our study may be the first to document that mats of *U. lactuca* kill softshells. Thiel et al. (1998) had similarly found that overgrowths of the filamentous alga *Enteromorpha prolifera* kill softshells in Maine; Breber (1985) found that mats of *Ulva rigida* and *Gracilaria* sp. kill carpet-shell clams, *Tapes decussatus*, in Italy; and Everett (1994) showed that the bent-nose macoma, *Macoma nasuta*, was more abundant in areas devoid of *Ulva expansa* than in areas where it formed mats in California. The same condition probably develops under *U. lactuca* mats that Gray (1992) described under *U. rigida* mats in Europe: Anaerobic conditions are reached and sulfide and other toxic compounds are produced leading to a massive mortality of benthic organisms.

Sarcoma infections occur seasonally (Farley 1976, Farley 1989, Cooper et al. 1982, Brousseau 1987, Barber 1990). Perhaps in collecting the softshells quarterly, we missed detecting some sarcoma in them. During most collections of adult softshells, a few recently dead specimens with whole shells were noticed among the 100–200 that were taken. Sarcoma might have been responsible for some mortality that was not identified to cause, or perhaps the softshells died from some other cause. We were unable to determine whether contaminants in the waters and sediments and dense phytoplankton blooms affected the longevity of the softshells. Barber (1990) found sarcomas in softshells in the Shrewsbury River in 1986 and 1987 and concluded that annual mortality due to the

disease was about 3.5% at that time. Our study cannot add much to his estimate.

The sizes of softshells at certain ages that Appeldoorn (1983) suggested for the Navesink River correspond with our findings in the Shrewsbury River. For example, at 20 months of age the softshells that Appeldoorn measured were 42.5 mm long and at 28 months they were 47.3 mm long, or similar to the mean lengths of softshells in the Shrewsbury River at about the same ages in November 1994 and June 1995. However, the comparisons are too crude to compare actual growth rates in the 1970s and the 1990s.

The small and sporadic commercial harvests of softshells in this area likely are due to their low setting densities and poor survival rates in recent years. The softshells probably would survive longer if a period of cooler summers and reduced eutrophication of waters were to follow.

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QUAHOG PARASITE UNKNOWN (QPX) IN THE NORTHERN QUAHOG *MERCENARIA MERCENARIA* (LINNAEUS, 1758) AND *M. MERCENARIA* VAR. *NOTATA* FROM ATLANTIC CANADA, SURVEY RESULTS FROM THREE MARITIME PROVINCES.

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ABSTRACT A histology based survey of 3047 quahogs from various sites in three Atlantic Canadian provinces between 1990–98 revealed Quahog Parasite Unknown (QPX) in clams ranging from 18–92 mm in length (> 1.5 years old). Prevalences ranged from 1.7% in wild quahogs to 80% in hatchery broodstock. An additional two year (1996–97) seasonal survey of four sites (St. Andrews and Shediac Bridge, New Brunswick; Wallace, Nova Scotia; and West River, Prince Edward Island) found QPX in quahogs 43–102 mm in length (n = 715) at Wallace (% P = 6.7%) and St. Andrews (% P = 20%). Infections were found in spring, summer and fall samples and no significant difference was found between male and female infected quahogs at either site (1996–97; χ^2, $P > 0.10$). The most commonly infected tissues were the gills, mantle and gonads.

KEY WORDS: Quahog Parasite Unknown (QPX), *M. mercenaria*, *M. mercenaria* var. *notata*, pathology

INTRODUCTION

Quahog Parasite Unknown (QPX) infects the hard-shell clam (northern quahog) *Mercenaria mercenaria* and the selected variety, *M. mercenaria* var. *notata* (Chanley 1961). It has caused cumulative mortalities ranging from 80% in quahogs from New Brunswick (Drinnan and Henderson 1963), and Cape Cod, Massachusetts (Smolowitz et al. 1998) to 100% in hatchery broodstock in Prince Edward Island (Whyte et al. 1994; Bacon et al. 1999). It also occurs in apparently healthy quahogs from Atlantic Canada and Virginia (McGladdery et al. 1993; Ragone Calvo et al. 1998). QPX has also been associated with quahog mortalities from Burton Bay, Virginia (Ragone Calvo et al. 1997). The Gulf of St. Lawrence is the northern-most limit of *M. mercenaria*, thus the clams may respond differently, both in terms of growth and disease resistance, from clams in the middle of their geographic distribution in the U.S. The conditions which trigger pathogenic infections levels, however, have yet to be determined.

Recent interest in developing the northern quahog for aquaculture in Atlantic Canada revealed a lack of base-line information on normal parasite and disease profiles for this species. Because culture involves handling and holding the clams in unnatural conditions, QPX has the potential to become a significant health problem, especially as hatchery broodstock are developed (Whyte et al. 1994). An accurate understanding of the seasonal and geographic distribution of QPX in wild and cultured populations throughout Atlantic Canada was, therefore, required. Throughout the past 10 years, samples of wild and cultured quahogs have been examined histologically for parasites and pathology, including QPX, as part of diagnostic services provided by Fisheries and Oceans Canada, Gulf Fisheries Centre, Moncton, New Brunswick. These data, in addition to a seasonal histological survey of wild quahogs from four sites in New Brunswick (NB), Nova Scotia (NS), and Prince Edward Island (PEI), conducted between 1996 and 1997, were examined to determine if there were significant population differences in quahog health profiles. Unlike other bivalve species cultured to date, quahogs in Atlantic Canada have undergone relatively little transfer and population mixing. This was, therefore, seen as an opportune time to collect base-line health information for subsequent development of the quahog aquaculture industry.

MATERIALS AND METHODS

Diagnostic Survey 1990–98

A total of 3047 quahogs was examined (Table 1). Quahogs (wild and *notata* variety) were collected or shipped live from aquaculture sites and hatcheries in NB, NS and PEI (Table 1) to the Gulf Fisheries Centre, Moncton, within 12–24 h of collection. Anterior-posterior length (mm) and weight (in shell) were measured before shucking. A 2–3 mm dorso-ventral cross-section was removed and fixed in 1% gluteraldehyde/ 4% formaldehyde (Howard and Smith 1983) for light microscopy. The tissues for light microscopy were paraffin embedded, sectioned (6 μm) and stained using Harris' hematoxylin and eosin.

Tissue sections were examined at 25 and 250 magnification using a Leitz Dialux 20 compound microscope. Prevalence of QPX was recorded, along with a qualitative scale for intensity of infection (light = < 25; organisms, moderate = 25–50 organisms; and heavy = > 50 organisms) per tissue section. The sex ratio of mature quahogs, infected with and without QPX, was compared to a 1:1 ratio using a standard Chi Square test (Zar 1984) to determine if there was any relationship between quahog sex and presence of QPX.

Seasonal Survey 1996–97

Wild quahogs were collected during the spring (May/June), summer (August) and fall (October/November) of 1996 and 1997 from: (1) St. Andrews, NB; (2) Wallace, NS; (3) West River, PEI; and (4) Shediac Bridge, NB (Figure 1). Samples of 28–30, total = 715, quahogs were collected and processed as described above, between May, 1996 and October, 1997 (Table 2). Water temperature and salinity were taken at the time of collection from all four sites during both years. In addition, a continuous temperature recorder was placed at the Wallace location from May to October 1996 and 1997. The sex ratio of infected quahogs was compared to a 1:1 ratio using a standard Chi-Square test (Zar 1984).

TABLE 1.

Collection details and QPX results for 1990–98 survey.

Date	Collection Site	Lengths examined (mm)	No.	Prev. (%)	**Inf. Levels	***Sex Ratio (Infected Quahogs)
1990	Ellerslie, PEI*	>25	5	80.0	H	4U
13-5-91	Bouctouche, NB	55–81	16	0	0	0
22-5-91	Shippagan, NB*	72–90	15	13.3	H	2M
17-6-91	Cocagne, NB	78–85	30	0	0	0
18-7-91	Cocagne, NB	75–89	30	0	0	0
29-7-91	Pictou, NS	83–104	45	0	0	0
6-7-91	Halifax, NS*	>25	10	0	0	0
15-8-91	Shediac Bridge, NB	73–91	30	0	0	0
17-8-91	Cocagne, NB	72–88	30	0	0	0
30-3-92	Shippagan, NB*	25–38	5	0	0	0
1-7-92	Cocagne, NB	71–91	30	0	0	0
26-8-92	Cocagne, NB	70–90	30	0	0	0
15-10-92	Cocagne, NB	45–63	30	0	0	0
7-6-93	Malagash, NS	57–76	30	0	0	0
22-6-93	Cocagne, NB	48–71	30	0	0	0
22-6-93	West River, PEI	54–73	30	0	0	0
13-7-93	Powell cove, NS	32–110	26	7.7	H	1M:1F
14-7-93	Wallace, NS	43–67	39	0	0	0
26-7-93	Brule Harbour, NS	53–180	30	3.3	L	1F
3-8-93	West River, PEI	52–82	30	0	0	0
4-8-93	Wallace, NS	49–62	30	0	0	0
8-8-93	Cocagne, NB	43–61	30	0	0	0
12-10-93	Ellerslie, PEI*	8–15	22	0	0	0
19-10-93	Malagash, NS	52–80	30	0	0	0
26-10-93	West River, PEI	51–96	30	0	0	0
7-6-94	West River, PEI	53–91	30	0	0	0
28-6-94	Cocagne, NB	65–75	30	0	0	0
12-7-94	Malagash, NS	52–63	30	0	0	0
22-8-94	West River, PEI	57–91	30	0	0	0
24-8-94	Cocagne, NB	63–71	30	0	0	0
13-9-94	Malagash, NS	54–84	30	0	0	0
2-11-94	West River, PEI	51–96	30	0	0	0
22-11-94	Malagash, NS	43–83	30	0	0	0
14-6-95	Shippagan, NB*	47–69	120	0	0	0
20-6-95	Shippagan, NB	<2	60	0	0	0
7-10-95	Shippagan, NB	<8	60	0	0	0
22-8-95	Bouctouche, NB	50–79	6	0	0	0
27-10-95	Little Harbour, NS* (n)	>25	8	2.5	H	1M
27-10-95	Ellerslie, PEI*	>25	4	0	0	0
14-5-96	Ellerslie, PEI	2–6	60	0	0	0
3-6-96	Ellerslie, PEI*	28–53	25	8.0	L	2M
11-6-96	Shippagan, NB* (n)	30–50	15	47.0	H	2M:4F:U
27-7-96	Ellerslie, NB*	30–40	6	0	0	0
27-7-96	Ellerslie, NB	<8	20	0	0	0
11-8-96	Little Harbour, NS*	72–105	60	0	0	0
29-8-96	Little Harbour, NS*	72–81	2	0	0	0
9-9-96	Orwell, PEI	3–7	150	0	0	0
11-4-97	Corkums Is, NS* (n)	33–67	26	31.0	H	5M:3F
7-6-97	Ellerslie, PEI	36–71	29	31.0	H	6M:3F
7-6-97	Vernon River, PEI	>25	30	0	0	0
9-6-97	Pugwash, NS	42–86	60	1.7	L	1M
9-6-97	Powell Cove, NS	45–75	60	0	0	0
9-6-97	Tatamagouche, NS	42–90	60	0	0	0
24-06-97	Shippagan, NB (n)	<10	48	0	0	0
20-10-97	Shemogue, NB (n)	7–22	121	0	0	0
20-10-97	Bouctouche, NB (n)	14–21	56	0	0	0
22-10-97	Vernon, R. PEI (n)	>25	30	0	0	0

TABLE 1.

Continued.

Date	Collection Site	Lengths examined (mm)	No.	Prev. (%)	**Inf. Levels	***Sex Ratio (Infected Quahogs)
22-10-97	Tatamagouche, NS (n)	>25	38	0	0	0
23-10-97	Baie Ste-Anne, NB (n)	>25	30	0	0	0
11-12-97	Ellerslie, PEI	28–33	30	0	0	0
15-01-98	Ellerslie, PEI* (n)	>25	25	0	0	0
15-01-98	Ellerslie, PEI*	>25	4	0	0	0
4-5-98	Little Harbour, NS*	70–98	60	0	0	0
8-5-98	Shemogue, NB (n)	<10	10	0	0	0
12-5-98	Bouctouche, NB (n)	<10	10	0	0	0
14-5-98	Ellerslie, PEI	<10	40	0	0	0
14-5-98	Ellerslie, PEI	<5	60	0	0	0
24-6-98	Ellerslie, PEI	29–95	60	6.7	H	1M:3F
26-5-98	Shediac Bridge, NB	89–102	6	0	0	0
5-6-98	St Cecile, NB (n)	<10	26	0	0	0
15-7-98	St Andrews, NB	44–87	40	10.0	H	3M:1F
30-7-98	Vernon River, PEI	18–25	30	6.7	M	2U
30-7-98	Vernon River, PEI (n)	20–25	30	0	0	0
4-8-98	Wallace, NS	<10	29	0	0	0
4-8-98	Wallace, NS (n)	<10	31	0	0	0
28-8-98	West River, PEI	80–100	30	3.3	M	1F
22-9-98	Shippagan, NB (n)	16–22	21	0	0	0
23-9-98	St Andrews	40–77	29	6.9	M	2M
6-10-98	St Cecile, NB (n)	11–24	40	0	0	0
9-10-98	St Cecile, NB (n)	17–27	19	0	0	0
13-10-98	Bouctouche, NB (n)	16–22	9	0	0	0
15-10-98	St Mary's Bay, NS	32–63	60	0	0	0
20-10-98	Shippagan, NB	13–30	60	0	0	0
20-10-98	Shippagan, NB (n)	15–20	23	0	0	0
26-10-98	Vernon River, PEI	19–25	31	42.0	M	4M:9U
26-10-98	Vernon River, PEI (n)	19–31	32	0	0	0
27-10-98	Baie de Vin, NB (n)	>25	45	0	0	0
27-10-98	Baie de Vin, NB	9–14	60	0	0	0
27-10-98	Percival River, PEI	43–63	30	3.3	M	1F
2-11-98	Wallace, NS (n)	7–16	27	0	0	0
2-11-98	Wallace, NS (n)	19–31	30	0	0	0
	Total		3047			

*- hatchery broodstock
(n) - *Mercenaria mercenaria* variety *notata*
**- H-heavy, M-moderate, L-light
*** - M-male, F-female, U-undetermined (resting/immature)

RESULTS

Diagnostic Survey 1990–98

No gross clinical signs were observed in any of the quahogs examined for the diagnostic survey, including clams with high intensities of infection detected using histological examination. QPX was found in *M. mercenaria* and *M. m.* var. *notata* from all three provinces. Prevalences ranged from 1.7% in wild quahogs from Pugwash, NS, in 1997, to 80% in moribund broodstock from the Ellerslie hatchery, PEI, in 1990 (Table 1). Of 3047 quahogs examined, 64 showed evidence of QPX infection (% P = 2.2) (Figure 2). Intensity of infection ranged from light to heavy. The size range of quahogs infected by QPX ranged from 18.3–92.5 mm (Table 1). The sex ratio of infected quahogs was 30 male:18 female:16 undetermined (resting stage or immature), which was not significantly different from 1:1 (χ^2, $P > 0.10$). The sex ratio of uninfected clams, however, was significantly different from 1:1 (996 male: 862 female: 1125 unidentified (resting stage or immature); χ^2, $P < 0.005$).

Of all the infected clams, the most commonly infected tissues were the gills (34%), mantle (31%) and gonads (31%) (Table 3). The digestive gland and foot were less commonly infected (12 and 5%, respectively).

Seasonal Survey 1996–97

No gross clinical signs were observed during necropsy of the quahogs collected for the seasonal survey. Clams from two of the four sites showed evidence of QPX infections: Wallace (1996 only) and St. Andrews (1996 and 1997) (Table 2). The summer sample of quahogs from Wallace had a prevalence of 6.7% QPX (light intensity). Quahogs from St. Andrews showed prevalences of QPX ranging from 3.3% (spring and fall, 1996), at light intensities, to 20% (summer 1997) at heavy intensities (Figure 3).

Figure 1. Map of Atlantic Canada showing sampling sites positive for QPX from all surveys and diagnostic material examined. The circle denotes the 1959–63 QPX study of Drinnan and Henderson (1963), diamonds denote the 1990–98 diagnostic survey, stars denote the 1996–97 survey and triangles show sites with QPX in hatchery broodstock. The dashed lines represents the northern-most limit of *M. mercenaria*.

The mean sample lengths of the quahogs examined (n = 715) ranged from 60.0 (± 10.7) to 83.5 (± 6.3) mm in 1996 and 61.9 (± 9.9) to 83.2 (± 4.9) mm in 1997. It was difficult to tell whether the same cohorts were sampled over the two year seasonal survey, because quahogs grow slower, once mature, in cooler northern waters than in warmer waters to the south. The highest water temperatures occurred in August at all sites and temperature ranges (8–24°C) were relatively consistent between sites for both years (Table 2). All four sites had moderate to high salinities (20–32‰) which were consistent over the survey period (Table 2). The highest prevalence (20%) was found in clams from St. Andrews in the summer of 1997 (Table 2). The second highest prevalence (13.3%) was found in the spring, 1997, sample. QPX was detected in one sample of clams from Wallace, in the summer of 1996 (6.7%). The temperature recorder on the Wallace bed recorded air temperatures, at low tide, as low as 0 °C in May, 1996 and as high as 34 °C in August, 1996 and 1997. The sex ratio of QPX-positive quahogs was 5 male:1 female in 1996 and 8 males:4 females in 1997, which was not significantly different from 1:1 (χ^2, $P > 0.10$, 1996 and $P > 0.25$, 1997). The sex ratio of uninfected clams was 173 male: 178 female: 1 unidentified (resting stage or immature) in 1996, and 187 male: 159 female: 1 unidentified (resting stage or immature) in 1997, which was not significantly different from 1:1 (χ^2, $P > 0.90$, 1996 and χ^2, $P > 0.10$, 1997). Of the infected clams, the most commonly infected tissues were the gonads (28%) and mantle (22%), although the digestive gland and foot (17%) and gills (11%) also showed high levels of infection (Table 3).

DISCUSSION

QPX or QPX-like organisms were first found in Atlantic Canada in the late 1950's/ early 1960's in wild *M. mercenaria* from Neguac, NB (Miramichi River estuary) in the Gulf of St Lawrence (Drinnan and Henderson 1963). Prevalences ranged from 50% in weak and dead quahogs to 5% in apparently healthy quahogs (Drinnan and Henderson 1963). Accumulated mortalities in grow-out tests conducted between 1959 and 1960 ranged from 60–90% in native quahogs to 20–25% in apparently healthy quahogs transplanted from nearby Miramichi beds (Drinnan and Henderson 1963). QPX was not investigated further until the early 1990's when it was found in moribund quahogs (15–30 mm in length) being conditioned for spawning at a hatchery in PEI (Whyte et al. 1994). The connective tissue and muscle were found to be infected with "an invasive eukaryote organism" identical to that described by Drinnan and Henderson (1963) and was given the non-taxonomic acronym "QPX" for "Quahog Pararsite Unknown" (McGladdery *et al.* 1993; Whyte et al. 1994).

QPX or QPX-like organisms have been found in quahogs from New Jersey in 1976 (Smolowitz et al. 1998) and more recently in quahogs from Virginia (Ragone Calvo *et al.* 1997, Ragone Calvo *et al.* 1998) and Massachusetts (Smolowitz *et al.* 1998). During the summer of 1995, 1.5–2 year old quahogs planted on aquaculture leases in Cape Cod, experienced mortalities with prevalences ranging from 10% in "non diseased" clams to 90% in diseased clams (Smolowitz et al. 1998). Cultured 1–2 year old clams (19–89 mm) from the eastern shore of Virginia ranged from 8–20% in 1996, to 4–48% in 1997, with associated mortalities estimated at 10–20% in the latter (Ragone Calvo et al. 1998).

The Miramichi Estuary of the Gulf of St. Lawrence is the northern-most geographic limit of *M. mercenaria*, thus QPX does not occur in the St. Lawrence River as mentioned in Ford et al. (1997) and Smolowitz et al. (1998). Prevalences of QPX in *M. mercenaria* and *M. m.* var. *notata* in the 1990–98 diagnostic survey ranged from 1.7% in *M. mercenaria* in Nova Scotia, to 80% in broodstock being conditioned for spawning at the Ellerslie Shellfish Hatchery, PEI (Table 1). No mortalities attributed to QPX have been found in wild quahogs in Atlantic Canada since the original cases reported by Drinnan and Henderson (1963), however, open-water mortalities are known to have occurred without being investigated (Drinnan, pers comm.). The highest prevalences of QPX recorded in the 1990–98 diagnostic survey were in both cultured native and *notata* variety broodstock from all three Maritime provinces (Table 1). The 1996–97 survey found 6.7% prevalence of QPX in clams from Wallace, N.S. and 3.3–20% QPX in an isolated native population at St. Andrews. Prevalences in quahogs at both sites were comparable to those found in US wild clams (8–90%, Smolowitz et al. 1998, Ragone Calvo et al. 1997 and 1998).

The size range of infected quahogs in this study ranged from 18 to 110 mm (Tables 1 and 2). Before 1998, the reported size range of QPX infected quahogs was > 35mm shell length. Despite their small size, the 18–25 mm cultured *M. mercenaria* from Vernon River, PEI, had been in the field for one year and were approximately 1.5 years old (Burleigh pers comm.). Ford et al. (1997) examined tissue sections of 2203 seed quahogs (< 1–20 mm and no more than a few months old) from 13 different hatcheries in six States. No evidence of QPX or QPX-like organisms was detected. QPX was also not detected in 756 hatchery-produced quahogs after a year of field grow-out (Ford et al. 1997), thus, it was

TABLE 2.

Collection details and QPX results for 1996–97 survey

Date	Collection Site	Water temperature (°C) and salinity (‰) at collection		Lengths examined (mm)	No.	Prev. (%)	**Inf. Levels	***Sex Ratio (Infected Quahogs)
2-5-96	Wallace, NS	8°	25‰	63–78	28	0	0	0
21-5-96	St Andrews, NB	10°	26‰	58–83	30	3.3	L	1M
5-6-96	West River, PEI	15°	25‰	69–91	30	0	0	0
11-6-96	Shediac Bridge, NB	15°	26‰	56–89	30	0	0	0
1-8-96	Wallace, NS	24°	30‰	65–78	30	6.7	L	2M
19-8-96	West River, PEI	22°	26‰	51–86	30	0	0	0
23-8-96	St Andrews, NB	22°	26‰	43–79	30	6.7	M	2M
27-8-96	Shediac Bridge, NB	23°	26‰	57–81	29	0	0	0
1-10-96	Wallace, NS	10°	20‰	64–84	30	0	0	0
17-10-96	West River, PEI	10°	23‰	52–91	30	0	0	0
21-10-96	St Andrews, NB	11°	26‰	43–76	30	3.3	H	1F
25-10-96	Shediac Bridge, NB	9°	30‰	73–95	30	0	0	0
27-5-97	St Andrews, NB	10°	25‰	53–87	30	13.3	L	3M:1F
5-6-97	Shediac Bridge, NB	14°	31‰	38–101	30	0	0	0
5-6-97	Wallace, NS	10°	26‰	51–88	30	0	0	0
9-6-97	West River, NS	16°	26‰	50–95	30	0	0	0
13-8-97	St Andrews, NB	21°	32‰	46–78	30	20.0	H	4M:2F
18-8-97	Wallace, NS	24°	31‰	66–93	30	0	0	0
26-8-97	West River, PEI	24°	29‰	50–93	29	0	0	0
29-8-97	Shediac Bridge, NB	21°	31‰	54–99	29	0	0	0
9-10-97	St Andrews, NB	10°	32‰	50–79	30	6.7	H	1M:1F
16-10-97	West River, PEI	8°	27‰	54–102	30	0	0	0
20-10-97	Wallace, NS	11°	32‰	71–93	30	0	0	0
24-10-97	Shediac Bridge, NB	10°	32‰	44–90	30	0	0	0
Total					715			

** - H-heavy, M-moderate, L-light

*** - M-male, F-female, U-undetermined (resting/immature)

concluded that hatchery-produced seed are unlikely to be infected by QPX. Conversely, Whyte et al. (1994) found QPX in infected hatchery-reared quahogs ranging from 15–30 mm in shell length. The report did not distinguish the exact size or age of infected quahogs, and no attempt was made to characterize the relationship between individual quahog size and presence of QPX (Whyte, pers. comm.). Due to colder growing conditions in the Gulf of St. Lawrence, compared with Massachusetts and Virginia, it is possible that the < 20mm quahogs examined by Whyte et al. (1994) could have been the same age as larger quahogs from further south. All QPX findings to date in the US have been from quahogs typically 1 to 2 years-old (Ragone Calvo et al. (1997 and 1998) and Smolowitz et al. (1998)).

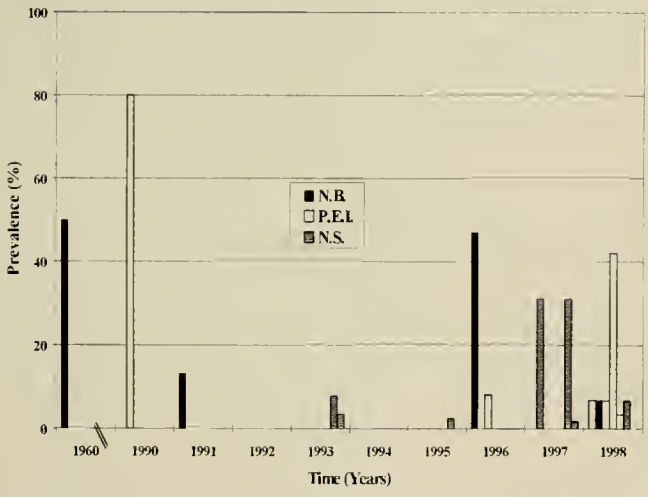

Figure 2. Historical and geographic summary of QPX in *M. mercenaria* and *M. mercenaria* variety *notata* from Atlantic Canada.

The taxonomic affinity of QPX is currently under investigation in both Canada and the U.S. (Smolowitz et al. 1998; Maas et al. 1999). Whyte et al. (1994) suggested that the QPX was similar to the labyrinthulids and thraustochytrids, belonging to the Phylum Labyrinthomorpha (Pokorny 1985). Members of these groups are common saprophytes in marine environments (Porter 1990), and have also been reported to cause disease in a number of molluscs

TABLE 3.

Prevalence of QPX in different tissues of infected quahogs.

Tissues	1990–98 percent of infected clams (n = 64)	1996–97 percent of infected clams (n = 18)
Gill	34	11
Mantle	31	22
Gonad	31	28
Digestive gland	12	17
Foot	5	17

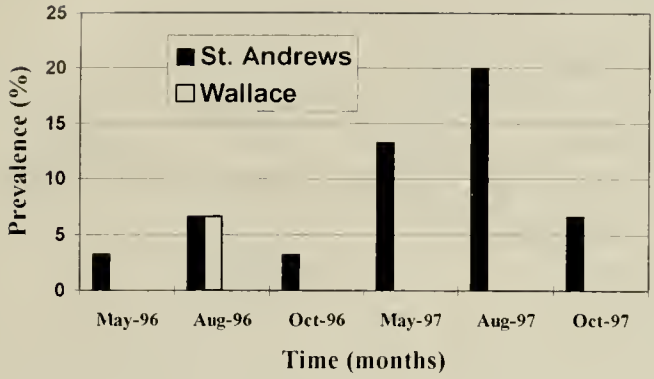

Figure 3. Prevalence of QPX from the two year repeated survey 1996–97. Solid black represents St. Andrews (Sam Orr Pond), N.B.) clams; unfilled box represent Wallace, N.S. clams. Clams from Shediac Bridge, N.B. and West River, P.E.I. were negative for QPX.

(Polglase 1980; McLean and Porter 1982; Jones and O'Dor 1983; Bower 1987a). One Labyrinthulid, *Labyrinthuloides haliotidis*, has been linked to mortalities of up to 100% of nursery-held juvenile abalone, *Haliotis kamtschatkana*, in British Columbia (Bower 1987a). Further investigation found that *L. haliotidis* is transmitted directly from abalone to abalone by a flagellated zoospore stage (Bower 1987b). Motile zoospores have been identified in both Canadian (Whyte et al. 1994) and U.S. (Kleinschuster et al. 1998) QPX cultures, therefore, it is likely that QPX is also transmitted directly. The likelihood of direct transmission would also be expected to be heightened in holding facilities or nurseries where clams are held in close proximity to each other in raceways, downwellers or upwellers. Further research on QPX transmission to both *M. mercenaria* and *M. m* var. *notata* is needed to fully understand the epizootiological potential of this parasite.

Smolowitz et al. (1998) noted thickened, retracted, light tan, swollen mantle edges in diseased clams from Cape Cod. Occasionally, yellow/tan nodules, 1–4 mm in diameter, were also observed along the mantle edges or in the mantle areas adjacent to the anterior adductor muscle. Shell margins were chipped and diseased quahogs showed variable amounts of sand embedded between the mantle edge and shell (Smolowitz et al. 1998). Smolowitz et al. (1998) postulated that shell chipping was a result of quahogs attempting to close their shells on the sand and sediment caught in the quahog's mucus. Soft tissues and shells were examined for all clams used in this study, however, no gross pathological changes have been seen, to date, in infected quahogs from Atlantic Canada, including heavily infected individuals.

Both sexes of quahog were infected with QPX . Prevalences in males were significantly higher than in females in the 1990–98 survey, but no significant differences were found between males and females in the 1996–97 seasonal survey. Uninfected quahogs examined in both surveys had a sex ratio of 1:1. No differences between the sex of infected clams have been reported elsewhere, to date.

The most commonly infected tissues in infected clams from the 1990–98 diagnostic survey were the gills (34%), mantle (31%) and gonad (31%). Similar results were found in the 1996–97 seasonal survey (gonad-28% and mantle-22%). Smolowitz et al. (1998) found that the most commonly infected tissues of infected quahogs from Cape Cod were the mantle (91%) and gill (63%). Ragone Calvo *et al.* (1998) also found the mantle (63%) and gills (35%) to be the most frequently infected tissues in infected quahogs from Virginia. The digestive gland (12–17%) and foot (5–17%) were less commonly infected in both 1990–98 and 1996–97 surveys. Smolowitz et al. (1998) also found the kidney (20–25%), adductor muscle (0–6%), foot (3–13%), digestive gland (0%), ganglia/mantle nerves (0%) and palps (0%) to be less heavily infected. Ragone Calvo *et al.* (1998) also observed infections in the musculature of the foot, sinuses and connective tissue of the kidney and connective tissue of the digestive glands (4, 11, and 15%, respectively). Drinnan and Henderson (1963) found QPX in the gill, kidney, connective tissue, foot, and heart of infected quahogs from New Brunswick but did not differentiate between levels of infection and tissue site. Although not quantified for this study, we found no evidence of palp, nerve or adductor muscle infections.

There are at least three environmental factors which may favour the proliferation of QPX in both hatchery and wild clams: i) stocking density; ii) water temperature; and iii) genetic susceptibility. Stocking density may have played an important part in the epizootic incident of QPX in wild quahogs from Neguac, N.B. (Drinnan 1961). The typical or natural stocking density of wild adult (> 20 mm) quahog populations in Atlantic Canada is approximately 4–5 clams m^{-2} (T. Landry, Fisheries and Oceans Canada, pers. comm.). Historically some quahog farming operations have planted seed (< 3 mm) at densities ranging from 357–43,011 m^{-2} (Judson et al. 1977; MacPherson et al. 1978; Witherspoon 1984) with no outbreaks of QPX reported. To date, only one report by Kraeuter et al. (1998) has examined the effects of planting density on proliferation of QPX. Juvenile quahogs (< 10 mm) from New Jersey were planted on intertidal and subtidal sites at three densities: 215, 430, and 860 clams m^{-2} per plot. The prevalence of QPX increased during the four-month experiment, but no significant effect, due to density or location, was detected (Kraeuter et al. 1998).

Water temperature and/or salinity may also be significant factors influencing the prevalence of QPX. All four sites in the seasonal survey experienced relatively similar temperature regimes, at the time of collection, ranging from 8 °C (May, 1996, and October, 1997) to 24 °C (August, 1996–97). Salinities ranged from 25–32‰ between 1996 and 1997. The clam beds at St. Andrews, Shediac Bridge and West River are all sub-tidal (1–3 m depth depending on tide level), whereas the Wallace site is completely exposed during each low tide. As a result, seasonal temperatures at the Wallace site, ranged from 8–28 °C (from May to October, 1996 and 1997), with air temperatures reaching as high as 34 °C at low tide. QPX was detected at the St. Andrews location in temperatures ranging from 10–22 °C and salinities ranging between 25–32‰. Historically, water temperatures at the St. Andrews site (Sam Orr Pond) range from –0.1–25 °C (Medcof 1961, S.M.C. Robinson, Fisheries and Oceans Canada, pers. comm.). The single QPX infection detected at Wallace occurred in August, 1996, when the water temperature was 24 °C and salinity was 30‰.

Ragone Calvo et al. (1998) collected quahogs from 18 different sites in Chesapeake Bay and coastal Virginia, where salinities ranged from 15 to 34‰. QPX was only detected in clams from three coastal lagoons, where salinities ranged from 30 to 34‰ (Ragone Calvo et al. 1998). Theses authors point out that the absence of QPX from more moderate salinities (15–25‰) may have been related to a limitation in QPX's salinity tolerance or have reflected sampling bias (Ragone Calvo et al. 1998). In seasonal collections from one Virginia coastal site, between July, 1996 and June, 1997, Ragone Calvo et al. (1998) observed the

highest prevalences and most severe infections in November and May samples. Smolowitz et al. (1998) reported that quahog mortalities in Massachusetts, associated with QPX infection, were highest in August and October. Temperature and salinity are known to be related to proliferation of other bivalve parasites such as *Perkinsus marinus*, *Haplosporidium costale* and *Haplosporidium nelsoni* (Bower et al. 1994, Ford et al. 1999), thus it is possible that QPX proliferation and pathogenicity may also be influenced by temperature and/or salinity.

Clam harvesting practices may also influence QPX proliferation. Harvesting of quahogs in Atlantic Canada has, traditionally, been done by hand (forks, tongs and rakes), although hydraulic harvesters are also used (Bourne 1989). The population of quahogs in Neguac, N.B., were harvested using an escalator harvester when mortalities started to increase, both in air storage and at Hay Island holding beds, between 1957 and 1959 (Drinnan 1960). Although no clear association between harvest methodology and QPX has been determined, its effect on physiological stress and defense capability seems worth investigating further.

In conclusion, QPX seems to be ubiquitous in both wild and cultured quahogs from the Maritime Provinces and is reported for the first time in quahogs from the Bay of Fundy. In light of past mortalities associated with this parasite, especially in hatchery broodstock being conditioned for spawning, QPX may present a significant challenge to development of quahog aquaculture in our region. The dynamics of infection and pathogenicity under different holding and handling conditions require more investigation to manage pathogen proliferation. This is especially important as uninfected populations seem to be few, if any, in Atlantic Canada, making selection of QPX-free broodstock an impractical solution.

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AGE AND SIZE OF *MERCENARIA MERCENARIA* IN TWO SISTERS CREEK, SOUTH CAROLINA

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ABSTRACT Northern quahogs, *Mercenaria mercenaria* (L.), were sampled from four sites in Two Sisters Creek, South Carolina. Shell lengths (SL) were measured and ages estimated from increments in shell sections. Mean SL of individuals collected at the two sites near the mouth of the creek were significantly larger than those collected in the upper reaches of the tidal creek. The back-calculated mean SL, however, were similar among sites within most age classes. Mean ages of individuals near the mouth were significantly older than those from the upper reaches. Differences in the population age structure were also observed among sites. Several factors are explored to explain the upstream pattern of decreasing SL and ages of quahogs in Two Sisters Creek.

KEY WORDS: *Mercenaria*, quahogs, clams, age, size, growth

INTRODUCTION

Commercial densities of northern quahogs, *Mercenaria mercenaria* (L.), occur in small creeks that dissect extensive tidal marshes of South Carolina and Georgia (Anderson et al. 1978, Walker 1987, 1989). Two Sisters Creek, South Carolina, which is representative of this type of habitat, became part of a State Shellfish Ground (SSG-134) in 1986. Reported landings from SSG-134, which also included Ashepoo River, Rock Creek, Atlantic Intracoastal Waterway, and Ashepoo-Coosaw Cut, averaged only 145 bags/year before an exploratory survey of quahog resources in Two Sisters Creek (S.C. Department of Natural Resources, unpubl. data). Resource managers perceived that quahog exploitation was limited before and after Two Sisters Creek became part of SSG-134. The mean (± SD) shell lengths (anterior posterior axis, SL) of quahogs collected during an earlier exploratory survey of May 6, 1987 indicated that individuals sampled from two sites nearer the mouth were larger (88.1 ± 10.37 mm and 87.1 ± 10.30 mm) than those collected from a mid-way site (69.0 ± 14.81 mm) and a site farther up Two Sisters Creek (61.6 ± 19.26 mm). A similar trend was observed in Christmas Creek, Cumberland Island, Georgia, where relatively higher numbers of larger quahogs (i.e., chowders) were found near the creek's mouth than in the upper reaches of the tidal creek (Walker 1987). Differences in quahog size among sampling sites could have resulted either from variations in growth rate or age of the respective populations.

The objectives of this study were to test the null hypotheses that growth rate and age were similar among quahog populations inhabiting four different upstream sites in Two Sisters Creek, South Carolina (Fig. 1). Age estimations, based on annual growth increments within the shell (e.g., Arnold et al. 1991, Jones et al. 1990, Peterson et al. 1985), were used to compare age and SL of different-aged quahogs from the four sample sites in Two Sisters Creek.

MATERIALS AND METHODS

Study Sites

Quahogs were sampled from four sites within Two Sisters Creek, South Carolina, on February 25, 1994. The site closest to the mouth was designated site 1, and sites 2, 3, and 4 were located progressively farther up the tidal creek (Fig. 1). Midchannel depths of the four sites at flood tide were 7.60 m, 8.20 m, 4.30 m, and 3.35 m, respectively. Tidal range was about 2 m. Bottom water temperatures and salinities at the time of collection ranged from 12 to 14 °C and 21 to 25 gl^{-1}. An estimate of bottom types indicated that sites 1, 2, and 3 were a mixture of mud, sand, and shell; whereas, site 4, although similar, seemed to contain more clay.

Sampling

Quahogs were collected at flood tide from subtidal sites with a hydraulic escalator harvester configured with a Maryland-type head. The mesh size of the escalator conveyor would retain quahogs >32 mm SL if not covered by mud or shell. In this event, smaller quahogs would be harvested. Subtidal bottoms at depths of 2–8 m were sampled across the width of the creek. Sampling at each site continued until sample sizes were ≥ 100 individuals. Quahogs were returned to Clemson University and frozen until analysis. Shell length and height (lateral axis, SH) were measured with calipers to the nearest 0.1 mm. After measuring, individuals were categorized according to the following commercial size groups: sublegals, < 44.4 mm SL; littlenecks, 44.4–67.9 mm SL; cherrystones, 68–78 mm SL; and chowders > 78 mm SL. A subsample of 50 quahogs per site, representative of the distribution at that site, was used for aging.

Age Determination

Quahogs were shucked, and the better valve was selected for sectioning. The valve of larger shells was cut from the ventral margin through the umbo, with a high-speed geological saw mounted with a diamond blade. Smaller shells were embedded in resin epoxy to avoid fracture during the sectioning (Kennish et al. 1980). Similarly, embedded shells were cut with a slow-speed saw mounted with a high-density diamond blade. Valves were polished with various grit carborundum papers and then etched in 1% hydrochloric acid. Age was obtained by counting translucent (dark) bands on the polished surface of a cut valve. Bands were counted

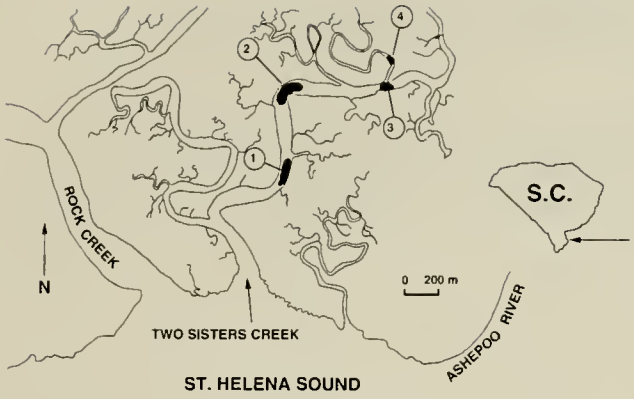

Figure 1. Sampling sites in Two Sisters Creek, South Carolina.

three times with two blind counts by the same observer. Values difficult to read were then washed and exposed to acetone before pressing against an acetate sheet (Kennish et al. 1980, Ropes 1984). The age of these clams was obtained by counting bands using a microfilm projector. A pattern of alternating translucent (dark) and opaque (light) increments on sectioned valves of known aged quahogs cultured in South Carolina waters was used to verify the formation of annual shell growth increments in the study (Devillers 1994).

Back-Calculated Shell Length

Shell heights from the umbo to the translucent increment for each age increment in the sectioned valves of shells were measured to the nearest 0.1 mm (see Jones et al. 1990). Measurements were limited to the first 12 increments, because of the difficulty associated with correctly measuring small increments thereafter. These measurements were then converted to SL using the equation (Eversole, unpubl. data):

$$\ln SL = 1.0493 \ln SH - 0.0136; r^2 = 0.997, n = 1{,}171.$$

Statistical Procedures

Analysis of variance (ANOVA) was used to determine significant differences in SL between the field sample and subsamples. Significant differences in age, SL, and back-calculated SL were also determined by ANOVA. Paired means were compared with the least significant difference (SAS 1985). Alpha level was set at 0.05 for these analyses.

RESULTS

Shell Lengths

Mean SL of the quahogs sampled from sites 1 and 2 were similar but significantly ($P \leq 0.05$) larger than those animals sampled at sites 3 and 4 (Table 1). Individuals from site 3 were also significantly larger than those quahogs sampled at site 4. The mean SL and ranges of these quahogs used for age determination were similar to that observed in the field sample (Table 1).

The frequency distributions of commercial quahog sizes collected from the four sites are presented in Figure 2. Chowders dominated the collections at site 1 (94.2%), site 2 (96.2%), and site 3 (69.8%). Site 4 contained 37.8% littlenecks and similar percentage of cherrystones (26.5%) and chowders (27.8%). Only 1.0%, 1.9%, 8.5%, and 7.9% of the quahogs were sublegal size (< 44.4 mm SL) in collections from sites 1, 2, 3, and 4, respectively.

TABLE 1.

Mean (± SD) and range (in parenthesis) of the shell lengths (mm) of *Mercenaria mercenaria* from four sites in Two Sisters Creek, South Carolina. Values in a column not sharing the same letter superscript are significantly different at $P \leq 0.05$. There was no significant difference between field and subsample mean SL.

	Field Sample		Subsample	
Site	N	Shell Length	N	Shell Length
1	104	93.27 ± 11.23[a] (41.9–110.7)	50	93.08 ± 12.68[a] (41.9–110.7)
2	104	94.27 ± 11.60[a] (36.0–117.5)	50	94.10 ± 11.32[a] (37.7–108.8)
3	106	79.05 ± 17.57[b] (32.2–101.9)	50	78.39 ± 17.81[b] (32.2–100.6)
4	151	67.84 ± 14.32[c] (33.2–97.6)	50	67.92 ± 14.59[c] (33.2–93.5)

[1] Site 1 was closest to the mouth while sites 2, 3 and 4 were progressively farther upstream in Two Sisters Creek.

Age

The oldest age of the sampled quahogs was 29 years from site 1, and the youngest was 1 year from sites 2 and 4 (Fig. 3). Significant differences ($P \leq 0.05$) were detected among the four sites in the average age of quahogs. Individuals on average (± SD) from site 2 (16.7 ± 4.70 years) were older than animals from site 1 (14.9 ± 5.37 years) and in turn, quahogs from site 3 (8.4 ± 3.96 years) and site 4 (4.7 ± 2.30 years) differed from each other and were significantly younger than those from sites 1 and 2. The range of ages in the sample from site 1 was 28 years with several (n = 10) unrepresented age classes in the distribution (Fig. 3). The distribution of ages from site 2 was similar (range and number of missing age classes) but differed from site 1 in having an obvious dominant age class at 15 years. The range of ages from site 3

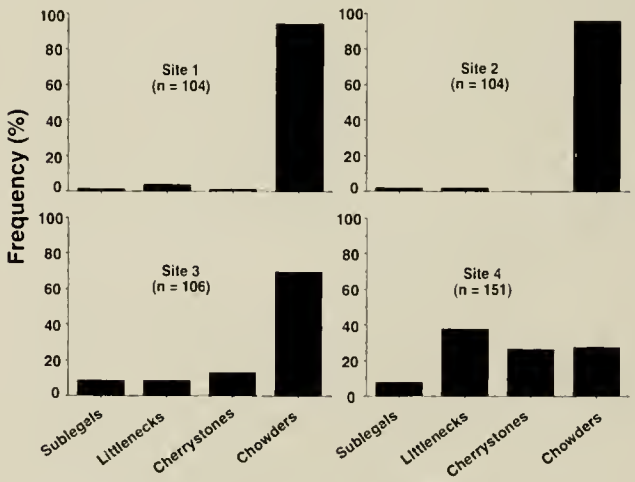

Figure 2. Relative frequency of commercial sizes of *Mercenaria mercenaria* from four sampling sites in Two Sisters Creek, South Carolina. Commercial sizes were sublegals (< 44.4 mm SL), littlenecks (44.4–67.9 mm SL), cherrystones (68–78 mm SL) and chowders (> 78 mm SL).

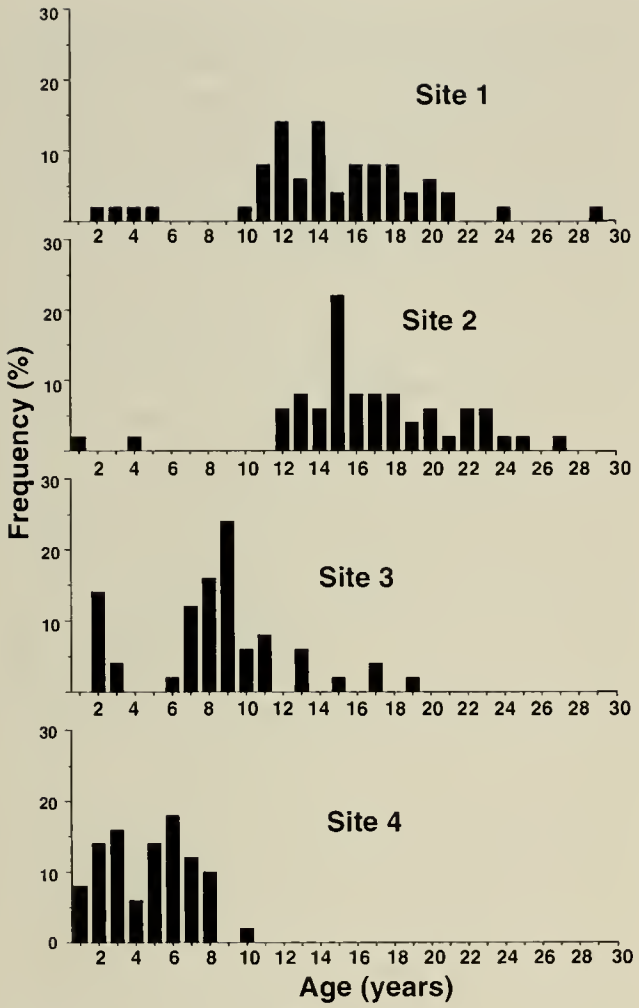

Figure 3. Age structure of 50 *Mercenaria mercenaria* collected from each of four sites in Two Sisters Creek, South Carolina.

encompassed 18 years with six missing age classes; whereas, those animals aged from site 4 spanned 10 years with only one missing age class at 9 years. None of the quahogs sampled from site 4 were older than 10 years; whereas, 90%, 96% ,and 22% of quahogs from sites 1, 2, and 3 were older than 10 years of age.

Back-Calculated Shell Lengths

The back-calculated mean SL of quahogs from sites 1, 2, and 3 were similar in size from age 1 through 11 years (Table 2). One significant difference among the four sites occurred from ages 1 to 4 years when quahogs from site 4 were significantly larger ($P \leq 0.05$) than those quahogs from sites 1, 2, and 3. The only other significant size difference in back-calculated SL occurred at 12 years (Table 2). Quahogs reached reproductive size (35 mm SL, Eversole in press) between 1 and 2 years and commercial size (44.4 mm SL) between 2 and 3 years.

DISCUSSION

Comparison of quahog sizes between this and other studies needs to be done with caution because of different sampling gear efficiencies and the common problem of the under representation of small individuals in samples (Fegley, in press). Although Walker (1987, 1989) used a different collection method, he did collect a similar range of sizes as those collected in this study and observed that chowders (> 78 mm SL) were the dominant commercial size class in 43% of the 40 sites sampled in Georgia waters. He also determined that chowders were more abundant in areas with little or no harvesting; whereas, littlenecks were more abundant in heavily fished areas. In a statewide survey of quahog habitat, littlenecks were found to be the most abundant commercial size class in South Carolina, which has a viable fishery (Anderson et al. 1978). In Two Sisters Creek, which has not been extensively harvested, the dominant commercial size was the chowder in three of the four sites sampled. Greene and Becker (1978), Malinowski (1985), Rice et al. (1989), and Walker (1989) have suggested that the gear used to harvest quahogs is biased towards the larger sizes resulting in differential removal of larger individuals and a shift in the population structure toward smaller commercial sizes. Conversely, it is anticipated that the larger commercial sizes would

TABLE 2.

Mean (± SD) of the back-calculated shell length (mm) by age (years) of *Mercenaria mercenaria* from four sites[1] in Two Sisters Creek, South Carolina. Values in rows not sharing the same superscript are significantly different at $P \leq 0.05$.

	Site 1		Site 2		Site 3		Site 4	
Age	N	Mean SD	N	Mean SD	N	Mean SD	N	Mean SD
1	50	27.4 ± 6.95^{a}	50	28.1 ± 6.73^{a}	50	27.3 ± 9.35^{a}	50	32.4 ± 6.60^{b}
2	50	47.2 ± 9.28^{a}	50	49.6 ± 9.09^{a}	50	47.4 ± 9.00^{a}	46	52.1 ± 8.31^{b}
3	49	61.8 ± 8.69^{a}	49	62.3 ± 7.86^{a}	44	63.4 ± 9.27^{a}	38	65.9 ± 8.07^{b}
4	48	72.2 ± 8.08^{a}	49	72.0 ± 6.72^{a}	41	74.3 ± 8.87^{a}	32	76.1 ± 8.54^{b}
5	47	79.4 ± 7.60^{a}	48	79.0 ± 6.23^{a}	41	80.4 ± 9.12^{a}	28	81.9 ± 8.99^{a}
6	46	83.8 ± 7.29^{a}	48	84.4 ± 6.75^{a}	41	84.8 ± 9.34^{a}	20	87.3 ± 8.15^{a}
7	46	88.2 ± 7.21^{a}	48	88.6 ± 6.85^{a}	39	88.6 ± 8.83^{a}	12	91.2 ± 8.72^{a}
8	46	92.0 ± 7.24^{a}	48	92.3 ± 7.22^{a}	34	91.8 ± 9.24^{a}	6	95.7 ± 8.23^{a}
9	46	95.8 ± 7.01^{a}	48	94.8 ± 7.16^{a}	26	93.2 ± 8.76^{a}	1	101.4
10	46	97.3 ± 7.49^{a}	48	97.5 ± 7.52^{a}	14	93.0 ± 6.79^{a}	1	102.3
11	45	99.9 ± 7.74^{a}	48	100.2 ± 7.76^{a}	11	95.4 ± 7.12^{a}		
12	40	101.7 ± 8.40^{ab}	48	102.5 ± 7.90^{a}	7	95.6 ± 8.45^{b}		

[1] Site 1 was closest to the mouth while sites 2, 3 and 4 were progressively farther upstream in Two Sisters Creek.

accumulate in areas not heavily harvested. This accumulation may explain why sites 1 through 3 in Two Sisters Creek were dominated by chowders (Fig. 2), but it does not adequately explain the dominance of littlenecks at site 4, which would be the least likely of the four sites to be commercially harvested or poached because of its size and location. In addition to the affect of harvesting, size and age structure of populations are also influenced by growth rates, recruitment, and mortality (Cerrato 1980). Because quahogs at site 4 grew at the same rate or faster than the quahogs at the other three sites, different growth rates can not solely be used as an alternative hypothesis for explaining differences in sizes among sampling sites.

Annual cycles of shell growth increment formation have been observed in shells of quahogs sampled from Rhode Island to Florida (Arnold et al. 1991, Fritz and Haven 1983, Jones et al. 1989, 1990, Kennish 1978, Peterson et al. 1985) and from South Carolina (Devillers 1994). Mean age determined from sectioned shells revealed that animals sampled from the sites nearest the mouth of Two Sisters Creek were significantly older than individuals collected from site 4 in the upper reaches of the tidal creek. Collections from sites 1 and 2 closest to the mouth also contained the oldest quahogs and the widest spread of ages. Differences in mean age and age frequency distribution among the sampling sites could have resulted from sampling error (e.g., small and under representative samples); however, on two separate sampling occasions and using the same gear, quahog size (age) decreased from sites near the mouth to upstream sites in Two Sisters Creek. If sampling error occurred, it was similar among sites and sampling occasions.

The absence of quahogs older than 10 years in the collection from site 4 may have been the consequence of a catastrophic event, intense predation or the recent successful establishment of the population. Low salinity periods resulting from hurricanes are reported to cause extensive quahog mortalities (Wells 1961). Although site 4 is more likely to be influenced by a catastrophic event than the other three sampling sites because of its smaller size, we have no evidence to indicate such an event occurred in this section of the coast 10 years ago. Furthermore, it is unlikely that such a large-scale event would have a stratified effect over such a restricted area as from site 1 to site 4.

The subject of settlement and postsettlement roles in defining macroinvertebrate soft-sediment communities has been extensively reviewed by Butman (1987), Olafsson et al. (1994), and Snelgrove and Butman (1994). Although these authors discuss several factors important in defining adult assemblages in soft sediments, it has not been clearly established whether adult spatial patterns result from differential larval settlement, differential postlarval survival, or redistribution (Armonies 1996, Bachelet et al. 1992, Peterson 1986, Wilson 1990).

Existing data indicate that hydrodynamic processes play a major role in determining the settlement of bivalves in soft marine sediments (e.g., see the review by Butman 1987). Near-bottom hydrodynamic forces determine the fate and flux of bivalve larvae over a patch of bottom. These forces are particularly important in the case with *M. mercenaria* larvae because of their weak swimming ability (Bachelet et al. 1992). *M. mercenaria* exhibited passive settlement when exposed to different sediment types in still and flume-flow water tests (Butman 1987, Butman et al. 1988).

Field studies evaluating the importance of hydrodynamics to recruitment are few (e.g., Carriker 1961, Mitchell 1974, Petersen 1986, Pratt 1953, Wilson 1990). Pratt (1953) provided the earliest suggestion that the distribution of quahogs was similar to the sediment particles in Narragansett Bay, Rhode Island, implying hydrodynamic processes were important in quahog distribution. He concluded from measurements of current patterns that early stage larvae coincided with the dense assemblages of adults and that hydrographic processes mixed and transported the larvae with time to potential settlement sites. Carriker (1961) commented that the most striking feature of the horizontal distribution of larvae in Little Egg Harbor, New Jersey, was its unevenness and as a consequence, quahogs set in areas that did not have adults. After studying quahog abundance and distribution in Southampton Waters, England, Mitchell (1974) came to a similar conclusion that the distribution of adult quahogs is in part controlled by tidal transport of the larvae produced by spawning beds. Mitchell (1974) also hypothesizes that variation in recruitment among years in different sites in Southampton Waters was related to the successful transport and settlement of competent larvae.

Andrews (1983) observed that most of the oyster larvae carried upriver during flood tide were transported down river during ebb tide with the exception of those few oyster larvae trapped upriver in oyster beds and small tidal creeks of James River, Virginia. Andrews (1983) also postulated that upriver entrainment was more successful in systems with low flushing rates than highly flushed systems. The four sites in Two Sisters Creek, because of their channel width and depth, have different flushing rates, with site 4 having the highest projected rate of the sites sampled. Quahog larvae produced in the main body of Two Sisters Creek probably could have been entrained in a tidal excursion at site 4. However, considering the patchy distribution of larval quahogs and the short window competent larvae have to set at slack tide (Carriker 1961, Armonies 1996), the probability of setting before being flushed from the small tidal creek was probably low. If entering and setting larvae survived predation pressures, perhaps a resident population of quahogs would have been established and served as a source of larvae for future recruitment at site 4. Because quahogs have a tendency to spawn at ebb slack water and be transported upstream with the subsequent flood tide (Carriker 1961), larvae from an established population at site 4 would have an increased probability of being retained in the tidal creek and recruiting to the population.

Another explanation for the different age distributions of quahogs in Two Sisters Creek involves the resuspension and distribution of postlarval individuals. Shifts from the initial distribution of recently settled *Macoma balthica* have been observed in the Wadden Sea (Armonies and Hellwig-Armonies 1992). Although postlarval *M. mercenaria* possess a temporary byssus thread (Carriker 1961), it can be released or broken resulting in dislodgment and resuspension by water flow (Butman et al. 1988). Resettlement of postlarval quahogs at site 4 in Two Sisters Creek would also require the appropriate hydrodynamic forces for transport and the subsequent survival of post larvae.

Predation helps shape quahog population structure (Bricelj 1993) by selecting the smaller (younger) more vulnerable individuals in the population (Whetstone and Eversole 1978, 1981). Of the suite of predators consuming quahogs (Gibbons and Blogoslawski 1989), crabs are the most important predators in South Carolina (Whetstone and Eversole 1978). Crab-related mortalities up to 100% were observed in juvenile quahogs planted in unprotected sites in Georgia and Florida (Menzel and Sims 1962, Godwin 1968). Quahog survival is improved if small individuals are provided some protection or if predators are removed (Eldridge et al. 1979, Peterson 1982). Greene and Becker (1978) observed an increase in quahog recruitment after a severe winter reduced the

number of blue crabs in Great South Bay, New York. Peterson (1982) demonstrated that the roots and rhizomes of seagrasses provide protection for infaunal species such as quahogs from some predators. Both Peterson (1982) and Wilson (1990) concluded that as much as 50% of the difference in quahog density between vegetated and unvegetated areas was attributable to enhanced post-larval survival. There is adequate information to indicate that post-settlement processes (predation) play an important role in invertebrate populations in soft marine sediments (see Olafsson et al. 1994 review). Although the distribution and abundance of predators within Two Sisters Creek could have played a role in the age distribution of quahogs among the four sites, we have no evidence to indicate predators either eradicated all the quahog sets 11+ years ago or selectively preyed on the older, larger individuals in site 4.

The maximum ages of quahogs are lower in faster-growing populations in southern latitudes along the United States coast than those observed in the slower-growing, more northerly populations of quahogs (Ansell 1968, Jones et al. 1990). Fewer quahogs would be expected to attain an older maximum age in site 4 if the faster-growing individuals at this site died at a younger age than at the other sites. Differential mortality of the faster-growing quahogs also helps explain why fewer chowders were observed in site 4 than in the other sites.

Our results illustrate that the differences in quahog sizes among sampling sites in Two Sisters Creek was attributable to different age structures at the four sites. Quahogs collected from the upper reaches of tidal creek (site 4) were younger than those collected downstream, and none of the quahogs from site 4 was older than 10 years; whereas, the oldest quahogs from sites 1, 2, and 3 were 29, 27, and 19, respectively. Although the first steps in establishing a population of quahogs involves settlement, the importance of hydrodynamic processes or predation (mortality) effects on post-larvae cannot be underestimated. Unfortunately, we have very little data to support a hypothesis to explain the observed age distribution in Two Sisters Creek. Future efforts to investigate quahogs recruitment should include an integrated approach that simultaneously considers factors such as hydrodynamic processes and post-settlement survival. Unraveling these causes of recruitment variation will be crucial to understanding the distribution and abundance of quahogs.

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MODELING GEODUCK, *PANOPEA ABRUPTA* (CONRAD, 1849) POPULATION DYNAMICS. I. GROWTH

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ABSTRACT In Washington State, target fishing mortality rates (F) for the geoduck clam, *Panopea abrupta* (Conrad, 1849), are based on relative changes in biomass and therefore depend on growth patterns. With these policies, higher growth rates lead to larger harvest quotas so that applying higher rates to areas with slower growth would cause overharvesting. Therefore, in estimating growth patterns, it is important to recognize the scale to which estimates of growth rates should be applied. In this study, we tested whether growth parameters differed among regions and among local sites within regions in Washington, and whether they differed enough to compel managers to create location-specific policies. Von Bertalanffy growth parameters were estimated for 11 sites dispersed among four regions. Among those sites, L_∞ ranged from 13.2 to 17.3 cm, k ranged from 0.113 to 0.235, and t_0 ranged from −0.029 to 0.806. Of the three parameters, the growth constant k had far more influence on target fishing mortality rates (F) than either L_∞ or t_0. Statistically significant differences in k were found among all local sites within geographic regions. However, only some of the differences were of a magnitude to concern management policies. We have proposed a general method for calculating and then testing for managerial significance when a linear relationship exists between k and the fishing mortality rate (F). Our results implied that managerially significant differences in k existed among local sites within Washington's geoduck management regions, posing a dilemma for managers who, by convention, propose a single target fishing mortality rate for each region.

KEY WORDS: Geoduck, growth, hypothesis testing, managerial significance, *Panopea abrupta*, von Bertalanffy

INTRODUCTION

The Pacific geoduck clam *Panopea abrupta* is a large hiatellid bivalve that occurs from Alaska to Baja, CA, and west to southern Japan (Bernard 1983). Geoducks are one of the largest burrowing clams in the world, reaching a live whole weight of 3.25 kg (Goodwin and Pease 1987). Adults are buried to 1 m in sand and mud substrates from the lower intertidal to depths of more than 110 m (Jamison et al. 1984). They dominate the biomass of benthic infaunal communities in many parts of Puget Sound, WA, where they have supported a commercial dive fishery in subtidal waters since 1970 (Goodwin and Pease 1991). Commercial dive fisheries also exist in Alaska and British Columbia (Campbell et al. 1998), and geoducks now provide the most valuable commercial clam harvest on the Pacific Coast of North America. The average annual ex-vessel value of Washington's geoduck harvest from 1990 to 1998 was US$14 million. From 1971 through 1997 annual landings have averaged 1,540 tons.

The Washington Department of Fish and Wildlife and several of the Washington tribes manage commercial geoduck harvest on a regional basis. There are six regions statewide that are based largely on legally defined tribal fishing boundaries. By coincidence, these boundaries also roughly conform to major oceanographic basins within Puget Sound (Ebbesmeyer et al. 1984). Currently, four of the regions (Fig. 1) are surveyed for biomass, and geoduck quotas are calculated annually for each of these regions as the product of biomass and a target fishing mortality rate. The target fishing mortality rate (F) is based on the output of an age-based equilibrium yield model (Bradbury and Tagart 2000), which relies in part on a three-parameter von Bertalanffy growth function. Past studies on geoduck growth (Goodwin 1976, Breen and Shields 1983, Anderson 1971) have only provided point estimates for annual growth increments, making it impossible to determine whether growth rates differed significantly among geographic areas. In this paper, we first estimated von Bertalanffy growth parameters for individual geoducks at 11 Washington sites and then conducted hypothesis tests for differences in growth parameters within and among the management regions.

In conducting a hypothesis test, statistical significance is not always biologically meaningful. In this study, statistical significance refers to *whether or not* the growth parameters change; biological significance refers to *how much* the growth parameters change. Statistical significance is well defined; however, biological significance is not. In this study, determining biological significance stemmed from the decision processes that were in place for managing the geoduck harvest and thus are more appropriately termed "*managerial significance*." Managerial significance was determined by how much the growth parameters must change before management decisions would be altered, and this degree of change was factored into the hypothesis-testing procedure. We concluded that according to the management criteria given, not only should regional specific growth parameter estimates be used, but within some regions site-specific estimates should also be used.

METHODS

Data

Geoducks were collected from 1979 to 1982 at 11 previously unfished sites in Washington (Fig. 1). The sites were chosen op-

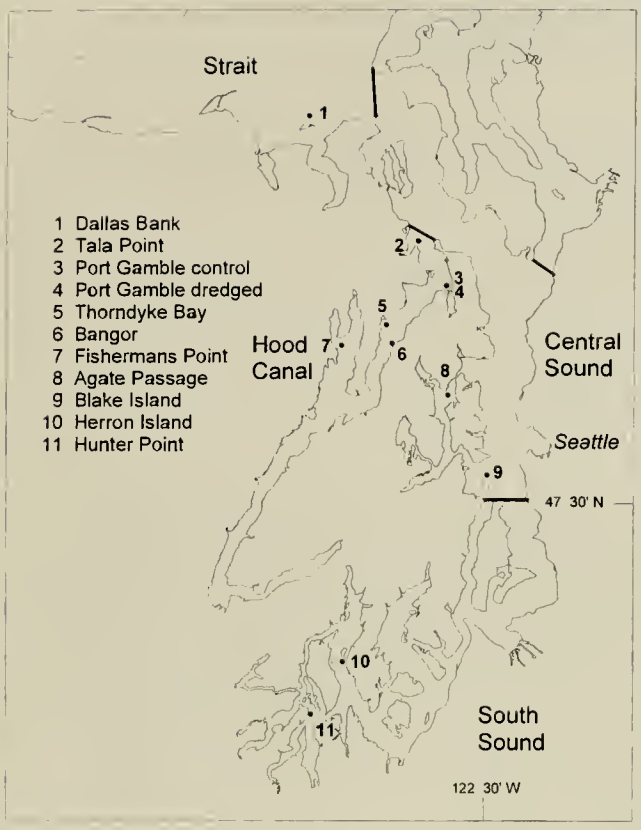

Figure 1. Sampling sites for geoduck growth. Also shown are boundaries for four of Washington's geoduck management regions; the two regions not shown contained no sampling sites and no surveyed geoduck biomass.

portunistically from among those scheduled at the time for prefishing surveys. However, they were spread out over the entire commercial fishing range of Washington geoducks. Preliminary dive surveys were conducted at each of the 11 study sites to map their boundaries. The shallow to deep boundaries of a commercial geoduck tract were set by management to be between 6 and 23 m mean lower low water. The along-shore boundaries of a commercial tract were subjectively defined on the basis of drops in geoduck densities, suitability of substrate, proximity to sewer outfalls or ferry traffic lanes, etc.

Geoducks were sampled from a series of transects. The transects were approximately 0.91 m wide, ran perpendicular to the shore from 6 to 23 m, and were spaced approximately 1 km apart (Fig. 2). In some of the larger sites, transect lines were spaced at systematic intervals wider than 1 km. In each site, the first transect was located opportunistically along the shoreline at one end of the mapped bed. Because the divers were unable to see either the substrate or the geoducks from the survey boat before selecting the starting point, we made the assumption that the selection represented a random starting point.

For logistical purposes, each transect was divided into 45.72-m-long subsections. Divers used a commercial water jet to dig geoducks from the approximate center of selected subsections. They were instructed to dig the first 10 geoducks seen without regard to size or any other criterion. The subsections selected were every fourth one, ignoring transect identity, i.e., as if the transects were laid end to end. For example, if the first two transects were each made up of 10 subsections, then the first, fifth, and ninth

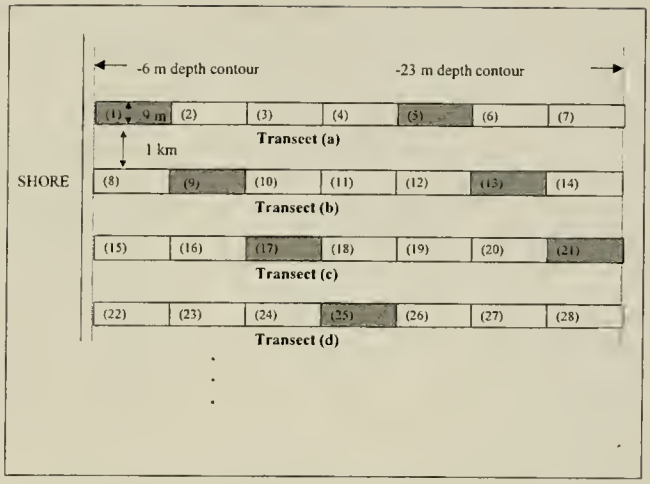

Figure 2. A schematic of the sampling design (not to scale or number). The smaller rectangles represent the hypothetical 46-m-long subjections (1–28) that make up the hypothetical transects (a–d). Ten geoducks were collected from every fourth subsection (shaded) as if the transects had been laid end to end.

subsections of the first transect would have been sampled and the third and seventh subsection of the second transect would have been sampled, etc. This procedure resulted in samples ranging from 21 to 258 geoducks, depending on the size of the site (Table 1).

A total of 1,216 geoducks were sampled from the 11 sites. They were held in saltwater and returned to the laboratory within a few days of collection for processing. All geoducks were numbered and separated by site before processing, and the greatest anterior-posterior length of the right valve was measured with calipers. Thirty geoducks per site were subsampled for this growth analysis. The subsample from each site was drawn randomly from the numbered shell samples, ignoring the subsection identity. Of the 330 sampled, 234 were used in the growth analysis. The 96 geoducks eliminated from the subsample were discarded either because they were unreadable or because they were <15 years old.

Annual growth increments were determined using the acetate-peel method developd by Thompson et al. (1980) and described for geoducks in Shaul and Goodwin (1982). Growth increments for ages beyond 25 years were not calculated, because geoducks reach their asymptotic size between the ages of 15 and 25 years (Shaul and Goodwin 1982). The yearly rings on individual geoducks were measured to provide length at age data for individuals. Thus, in the rth region ($r = 1 \ldots 4$), the data for the ith individual ($i = 1 \ldots n_r$) consisted of paired observations (l_{ijr}, a_{ijr}, $j = 1, \ldots J_{ir}$) where l_{ijr} was the length measured for age ring j and a_{ijr} was the age assigned to age ring j. The number of individuals varied by region and the number of age rings varied by individual (because some geoducks were less than 25 y old). From the paired observations on each individual, length was regressed on age with a nonlinear von Bertalanffy function:

$$l_{ijr} = L_{\infty,ir}(1 - e^{k_{ir}(a_{ijr} - t_{0,ir})}) + \varepsilon_{ijr}$$

where $\varepsilon_{ijr} \sim N(0,\sigma^2_{ir})$. Thus, each nonlinear regression produced a set of estimated parameter values $\{\hat{L}_{\infty,ir}, \hat{k}_{ir}, \hat{t}_{0,ir}\}$ for the ith individual ($i = 1 \ldots n_r$) in the rth region ($r = 1 \ldots 4$). The variance σ^2_{ir} represented both the measurement error and the uncertainty caused by the absence of old growth rings. Therefore, the vari-

TABLE 1.

Sample size, mean shell length and von Bertalanffy growth parameter estimates (±SE) derived from shell length at age for *P. abrupta* at 11 sites in Washington.

Region	Site	No. Dug	No. Subsampled	Mean Shell Length (cm)	L_∞ (cm)	k	t_0
South Sound	Hunter Point	71	21	15.2	16.4 (± 0.357)	0.2283 (± 0.009)	0.719 (± 0.040)
	Herron Island	36	23	12.5	13.2 (± 0.158)	0.1544 (± 0.006)	0.422 (± 0.074)
Central Sound	Agate Passage	208	20	13.6	15.8 (± 0.383)	0.1964 (± 0.009)	0.183 (± 0.066)
	Blake Island	19	18	13.0	14.6 (± 0.283)	0.1586 (± 0.006)	0.806 (± 0.071)
Hood Canal	Bangor	98	25	13.5	14.3 (± 0.252)	0.1569 (± 0.007)	0.545 (± 0.055)
	Tala Point	96	24	12.4	13.6 (± 0.361)	0.1435 (± 0.009)	−0.029 (± 0.071)
	Port Gamble (dredged)	180	21	13.1	15.2 (± 0.283)	0.1810 (± 0.007)	0.661 (± 0.052)
	Port Gamble (control)	80	21	12.7	14.0 (± 0.390)	0.1610 (± 0.007)	0.599 (± 0.075)
	Thorndyke Bay	258	21	12.2	13.0 (± 0.201)	0.1421 (± 0.005)	0.550 (± 0.097)
	Fishermans Point	21	19	16.8	17.3 (± 0.251)	0.2353 (± 0.009)	0.552 (± 0.059)
Strait	Dallas Bank	149	21	12.0	13.3 (± 0.405)	0.1131 (± 0.005)	0.334 (± 0.096)

ances for older geoducks with more growth rings were likely to be more precise than for younger geoducks. The resulting heteroscedasticity for geoducks >15 years of age was thought to be minimal and was ignored.

Hypothesis Testing

The experimental design was a two-factor analysis of variance (ANOVA) in which the first factor was region and the second was sites nested within regions. We first conducted a hypothesis test on site effects within each region and only conducted a test among regions if the site effects were nonsignificant. In this case, nonsignificance meant not managerially significant. Thus, for all statistically significant tests, Tukey multiple comparisons (Neter et al. 1985) were used to test for managerial significance. The Tukey multiple comparisons yielded confidence intervals for the differences in growth among locations. For any one comparison to be managerially significant, the difference in growth had to be at least some constant c. These comparisons were identified by confidence intervals that excluded the interval $(-c, c)$.

Calculating Managerial Significance ($c = 0.027$)

Most U.S. and Canadian fisheries, including all of those under U.S. federal jurisdiction, are managed using biological reference points (BRPs). BRPs are calculable quantities that describe a population's state and are usually used as targets for optimal fishing (National Research Council 1998). A BRP is most often expressed as a fishing mortality rate (F); examples include F_{MSY}, F_{max}, and $F_{xx\%}$. These are the fishing mortality rates that are expected to achieve, over the long term, maximum sustainable yield, maximum yield per recruit, and a spawning stock biomass that is $xx\%$ of the unfished level, respectively.

We considered two management criteria in calculating the threshold of managerial significance for geoduck growth parameters: (1) the BRP used by managers in setting the target fishing mortality rate and (2) the number of significant digits to which this target fishing mortality rate was calculated. Geoduck managers in Washington currently use as a BRP the fishing mortality rate corresponding to $F_{40\%}$, a reference point that is widely used for U.S. West Coast groundfish (Clark 1993). Managers have agreed to calculate this target fishing mortality rate to three decimal places. For example, there is a managerially significant difference between annual fishing mortality rates of 0.027 and 0.028, but not between 0.027 and 0.0273.

The three von Bertalanffy growth parameters were first evaluated to determine which had the most influence on yield model predictions. The equilibrium model described in Bradbury and Tagart (2000) was used to calculate $F_{40\%}$ for different values of $\{L_\infty, k, t_0\}$ in the range observed in the data. L_∞, while it affected model predictions of absolute yield, did not affect relative spawner-per-recruit biomass or relative yield per recruit and was therefore eliminated from further analysis. Figure 3 shows that the growth parameter k is more influential on $F_{40\%}$ than t_0. Because L_∞ had no impact on $F_{40\%}$ and t_0 had only minimal impact, we conducted univariate hypothesis tests on k.

Changes in k would only affect management decisions if they caused the model-based fishing mortality rate to change by 0.001 or more. In general, whenever a linear relationship exists between k and F with slope β,

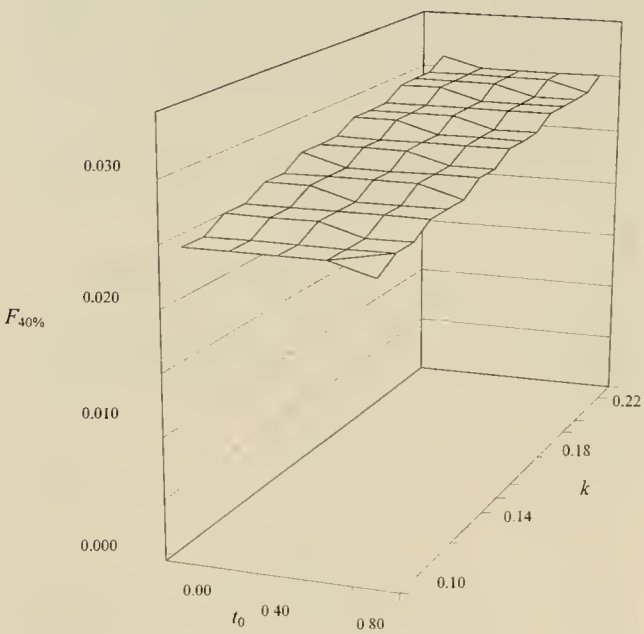

Figure 3. Surface plot of $F_{40\%}$ values as a function of the observed ks and t_0s.

$$\frac{\Delta F_x}{\Delta k} = \beta \Rightarrow \Delta k = \frac{\Delta F_x}{\beta}.$$

In this example, a linear regression of the level of change $F_{30\%}$ on k yielded a highly significant slope ($P < 0.001$) of $\beta = 0.0366$. Using this slope and Washington's management decision to calculate annual fishing mortality rates to three significant digits, $c = 0.001/0.0366 = 0.027$. Thus, absolute differences in k among sites (or regions) ≥ 0.027 were managerially significant.

RESULTS

Table 1 shows the von Bertalanffy parameter estimates and their variances for the 11 study sites. The resulting growth curves for the fastest-growing site (Fishermans Point) and the slowest-growing site (Dallas Bank) are shown in Figure 4; the growth curves for all other sites lie between these two. Also shown for comparison is Anderson's (1971) growth curve for geoducks at Big Beef Creek and Dosewallips beaches in Hood Canal.

Test for Nested Site Effects

The test for site effects within regions was statistically significant ($F_{7,223} = 24.72$, $P = 0^+$) for all regions. Further testing for managerial significance produced mixed results. The Tukey multiple comparisons between sites within regions showed four comparisons in which the differences were managerially significant (Table 2): between the sites in the South Sound region and among several sites in the Hood Canal region.

Power of Tukey Multiple Comparisons

Because the null hypothesis of the growth parameters not being significantly different was not rejected for sites within Hood Canal and for the two sites in the Central Sound region, we conducted a power analysis to assess whether or not the nonrejection was meaningful. The power analysis estimated the probability that any one of the Tukey multiple comparisons would have excluded the interval (−0.027, 0.027) if in fact the differences in k among sites had been at least 0.027. To estimate this probability, we used the two-sample t-test power analysis option of Power Analysis and Sample Size (PASS version 6.0, Hintze, 1996). Each of the multiple comparisons was a t-test that needed a Tukey multiplier. To adapt the software into giving the appropriate power estimates (Table 3), we inflated the estimated standard deviation of the comparison, ($\sqrt{MSE} = 0.0309$, calculated by the ANOVA) by the ratio of the Tukey multiplier (3.217) to the analogous Z multiplier (1.96):

$$\sqrt{MSE}_{\text{inflated}} = 3.217/1.96\sqrt{MSE} = 1.64\sqrt{MSE} = 0.0507.$$

Power in the Hood Canal Region

In the Hood Canal region, there were 6 sites and 15 comparisons, 3 of which were significant. With an average sample size of 22, the estimated probability was 0.4233 (Table 3) for detecting a 0.027 difference in any one of the comparisons. If the actual differences in k among sites had been at least 0.027, then one would expect to detect more than three of them. In fact, the probability of detecting a difference of 0.027 in at most three comparisons, where the probability of detection was 0.4233 per comparison, is the probability that a binomial random variable with $N = 15$ and $P = 0.4233$ was less than or equal to 3. This probability was 0.0645. Given that this probability was very low, there is evidence that among the sites in Hood Canal, other than Fishermans Point, the differences in k are not likely to be greater than 0.027 and thus need not be estimated separately.

Power in the Central Sound Region

In the Central Sound region, there were two sites, and the comparison was not significant. With a power of 0.4233 of detecting significance in a comparison, the chance of not rejecting the null hypothesis was $1 - 0.4233 = 0.5767$. Because this probability is high, nonrejection of the null hypothesis was not meaningful; i.e., the results are inconclusive.

Because Hood Canal was the only region producing evidence for common growth rates among sites, we did not pursue a test of regional differences. Thus, we recommend that with the given management criteria, separate growth models should be used in the regions given in Table 4. For the sites within Hood Canal other than Fishermans Point, the average growth parameter was calculated as the mean of the average growth parameters in each site (Table 4).

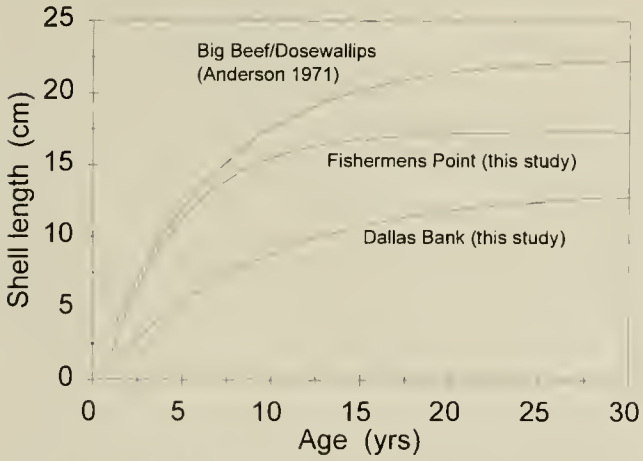

Figure 4. The von Bertalanffy growth curves for geoduck growth at the fastest growth site (Fishermans Point) and the slowest growth site (Dallas Bank) in this study. Also shown is Anderson's (1971) growth curve for Big Beef/Dosewallips.

DISCUSSION

The first result to note is the difference in the growth curves presented here and that from Anderson (1971). We estimated both a lower rate of growth (k) and a smaller asymptotic size (L_∞) for geoducks; however, differences in the target population explain this discrepancy. Anderson's target population consisted of subtidal and intertidal geoducks between the presumed ages of 1 and 5 years. Our target population consisted of subtidal geoducks older than 15 years. Because mean geoduck shell length is inversely proportional to water depth (Goodwin and Pease 1991), it is expected that Anderson's sample would have a higher estimate of L_∞. Likewise, a higher estimate of k is expected with a younger target population.

Of the three von Bertalanffy growth parameters, only one was determined to be influential: the parameter k. For the criteria given, managerial significance was calculated to be differences in k of 0.027 or greater among locations. That is, if the growth parameter differed by more than 0.027 among locations, then location-specific growth estimates should be used for setting harvest quotas.

Data that were collected in four different regions encompassing

TABLE 2.

Confidence intervals for the multiple comparisons of Test 1.

Site	Comparison (x_1/x_2)	$\Delta = x_1 - x_2$	SD(Δ)	Lower Bound*	Upper Bound
South Sound	Herron/Hunter†	−0.0739	0.0102	−0.1164	−0.0314
Central Sound	Agate/Blake	0.0379	0.0109	−0.0076	0.0834
Hood Canal	Bangor/Tala	0.0133	0.0088	−0.0151	0.0417
	Bangor/Gamdredge	−0.0242	0.0092	−0.0536	0.0053
	Bangor/Thorndyke	0.0147	0.0092	−0.0147	0.0442
	Bangor/Gamcontrol	−0.0042	0.0092	−0.0336	0.0253
	Bangor/Fishermans Point†	−0.0784	0.0094	−0.1087	−0.0481
	Tala/Gamdredge	−0.0375	0.0092	−0.0672	−0.0077
	Tala/Thorndyke	0.0014	0.0092	−0.0283	0.0311
	Tala/Gamcontrol	−0.0175	0.0092	−0.0472	0.0123
	Tala/Fishermans Point†	−0.0917	0.0095	−0.1223	−0.0612
	Gamdredge/Thorndyke	0.0389	0.0095	0.0082	0.0696
	Gamdredge/Gamcontrol	0.0200	0.0095	−0.0107	0.0507
	Gamdredge/Fishermans	−0.0542	0.0098	−0.0858	−0.0228
	Thorndyke/Gamcontrol	−0.0189	0.0095	−0.0496	0.0118
	Thorndyke/Fishermans†	−0.0931	0.0098	−0.1247	−0.0616
	Gamcontrol/Fishermans†	−0.0743	0.0098	−0.1058	0.0428

* The confidence intervals were calculated using a Tukey multiplier of 3.217, i.e., $\Delta \pm 3.217 * SD(\Delta)$. The multiplier corresponded to a studentized range distribution 95th% quantile with 11 and ∞ degrees of freedom.
† Statistically significant data in these rows.

11 different sites were tested for differences among growth parameters. Statistically significant differences in k were detected among most of the sites within the three regions Central Sound, Hood Canal, and South Sound. Further testing showed that in the South Sound, the sites were also significantly different. In Hood Canal, only one site was significantly different from the others. In the Central Sound, the results were inconclusive. Therefore, to preserve the management sensitivity criterion of 0.001 in the estimated $F_{40\%}$ levels, we recommend different growth parameter estimates be used for each site in Straight, Central Sound, and South Sound and that one common model for the sites in Hood Canal other than Fishermans Point be used.

We speculate that environmental factors related to tidal flow may have been a primary cause of the differential growth rates. Goodwin and Pease (1991) found that the average shell length of geoducks in Puget Sound was greatest in sandy substrates and decreased in both muddier substrates and those composed of pea gravel. Because size and growth are related, it is reasonable to conclude that growth is greatest in sites that are subject to intermediate tidal flow (i.e., those composed primarily of sand) and decreases in both low-energy (muddy) and high-energy (gravelly) environments. The substrate was primarily composed of sand at the three sites in our study with the highest k values (Fishermans Point, Hunter Point, and Agate Passage). The three sites with the lowest k values were Dallas Bank, a site composed primarily of pea gravel, and Tala Point and Thorndyke Bay, both of which are muddy. Goodwin and Pease (1991) also suggested relationships betwen geoduck size and environmental factors such as primary productivity and water temperature. Along with tidal currents, these factors are likely to vary from site to site, resulting in differential growth parameters.

Evidence for site-specific growth differences poses a dilemma for managers who must recommend a single regional harvest rate. If growth rates were common among sites, a regional estimate based on any selection of sites would be unbiased. However, we found that the growth constant can be site specific, requiring ad-

TABLE 3.

Power estimates for a single Tukey multiple comparison for various sample sizes.*

Sample Size	Power
20	0.3914
21	0.4075
22	0.4233
23	0.4389
24	0.4542
25	0.4693
30	0.5409

* Power was estimated using the two-sample T-test option of PASS version 6 (Hintze 1996) with a standard deviation of 0.0507 and a difference in means of 0.027.

TABLE 4.

Growth Parameter k estimated by region and site.

Region	Site	Estimated k
South Sound	Hunter	0.2283
	Herron	0.1544
Central Sound	Agate	0.1964
	Blake	0.1586
Hood Canal	Bangor	0.1569
	Tala	0.1569
	Gamdredge	0.1569
	Thorndyke	0.1569
	Gamcontrol	0.1569
	Fishermans Point	0.2353
Strait of Juan de Fuca	Dallas	0.1131

justments to a sampling plan for estimating an unbiased regional parameter. Because the sites in this study were not selected at random, a regional k that is an average of the estimated site ks will be biased. Managers might consider using the lowest estimated k-value with the expectation that that would be a conservative approach. Alternatively, another study could be conducted using a sampling plan designed to yield unbiased regional estimators.

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MODELING GEODUCK, *PANOPEA ABRUPTA* (CONRAD, 1849) POPULATION DYNAMICS. II. NATURAL MORTALITY AND EQUILIBRIUM YIELD

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ABSTRACT The natural mortality rate of geoduck clams, *Panopea abrupta* (Conrad, 1849), was estimated from data collected at 14 previously unfished sites in Washington State in order to predict the potential yield of the commercial fishery under various harvest rate strategies. The instantaneous rate of natural mortality (M) estimated by the catch curve method for geoducks of ages 28–98 was 0.0226 y^{-1}. Other important life history parameters—growth, schedules of sexual maturity, weight-at-age, and fishery selectivity—were estimated from the literature and file data. These parameter estimates were used to drive an age-based equilibrium yield model that predicted yield per recruit (YPR) and spawning biomass per recruit (SPR) over a range of fishing mortality rates. The model produced values of the instantaneous fishing mortality rate (F) for five commonly used constant harvest rate strategies. The fishing mortality rate producing maximum YPR (F_{max}) ranged from 0.053–0.100 depending on the site growth parameters, but reduced SPR to 15–21% of the unfished level. F-values for the $F_{0.1}$ strategy ranged from 0.28 to 0.37, reducing SPR to 35–37% of the unfished level. Three harvest rate strategies that reduce SPR to either 35%, 40%, or 50% of the unfished level were also evaluated, with F-values ranging from 0.018 to 0.036. The $F_{40\%}$ strategy, currently adopted by Washington managers, was achieved with $F = 0.028$ (averaged over all sites), corresponding to an annual harvest rate of 2.7% of the exploitable biomass. The model was most sensitive to estimates of M, whereas growth, fishery selectivity, and sexual maturity schedules had relatively little effect on yield or SPR. Apparent shifts in recruitment during the past 30–45 y may have biased the estimate of M. Direct estimates of M and recruitment are therefore a high research priority if the model outputs are to remain useful.

KEY WORDS: Geoduck, *Panopea abrupta*, natural mortality, yield, harvest rate, spawning biomass

INTRODUCTION

The geoduck clam *Panopea abrupta* (Conrad, 1849) supports the most economically important clam fishery on the Pacific Coast of North America (Campbell et al. 1998, Hoffmann et al. 2000). Since 1967, the Washington Department of Fish and Wildlife has performed annual dive surveys to estimate the exploitable biomass of geoducks in Washington. "Exploitable biomass" here refers only to geoducks within the legally fishable water depths of 6–23 m, in areas that are not polluted or otherwise unsuitable for commercial fishing and of sufficient size for their siphons to be visible to divers. Based on market and survey samples in Washington, this excludes most geoducks <300 g. Of 11,181 geoducks randomly sampled using commercial methods, only 2% were <300 g (Goodwin and Pease 1987). Geoducks usually attain this size in 5–7 y (Hoffmann et al. 2000).

Exploitable geoduck biomass on a commercial bed is estimated as the product of the total bed area, the mean weight per geoduck, and the mean density of geoducks. Mean density is estimated by counting geoduck siphon "shows" using a systematic strip transect technique (Goodwin 1978). Mean weight per geoduck is estimated from a series of samples dug at systematic intervals along the transect lines. The sum of the most recent biomass estimates on all surveyed beds within a management region constitutes the regional biomass estimate. There are currently six geoduck management regions in Washington, based largely on legally defined tribal fishing boundaries. Because only a few beds can be surveyed intensively in this manner each year, regional biomass estimates consist of the most recent estimate for each bed, with known catches subtracted from those beds as they are fished.

To establish annual fishing quotas, managers apply a target harvest rate to the exploitable biomass estimate in each management region. Beginning in 1981, the target harvest rate was fixed at 2% of the estimated virgin (unfished) biomass on surveyed, commercially viable beds. This target harvest rate was based on a Ricker (1975) yield per recruit (YPR) model, but the model outputs were never explicitly documented. Furthermore, emphasis in fisheries management has shifted since that time; harvest strategies based on YPR analyses (e.g., F_{max} and $F_{0.1}$) are now often supplanted by strategies that seek instead to preserve the reproductive potential of the population. These spawning biomass per recruit (SPR) strategies are increasingly being used in marine finfisheries (Clark 1991) and to a lesser extent in shellfisheries (Quinn and Szarzi 1993).

In this study, we derive estimates of the natural mortality rate (M) from geoducks sampled at previously unfished sites in Puget Sound and the Strait of Juan de Fuca. We also construct schedules of sexual maturity, weight-at-age, and fishery selectivity from the literature and file data. We use these estimates to drive an age-based equilibrium yield model that predicts YPR and SPR over a range of fishing mortality rates. We also explore the limitations of the model and conduct sensitivity tests to determine which parameters most influence the model's predictions. Finally, we use this information to recommend research aimed at refining the most important parameter estimates.

METHODS

Sampling Sites and Procedures

Geoducks were sampled between 1979 and 1981 at 14 previously unharvested sites in Puget Sound and the Strait of Juan de

Fuca to obtain information on age distribution (Fig. 1). The sites span four of the current six management regions, with six sites in the Hood Canal region, two sites in the Central Sound region, one site in the Strait region, and two sites in the South Sound region. Samples were taken randomly within each site at depths of 10–20 m by washing geoducks from the substrate with a commercial water jet. Age was determined from annual growth increments in the hinge plate using the acetate-peel method (Shaul and Goodwin 1982).

The instantaneous rate of natural mortality (M) was estimated from the geoduck age-frequency distribution using two different catch curve models (Robson and Chapman 1961, Ricker 1975). Both models assume that mortality is constant for all ages used in the catch curve. The Robson and Chapman model is based on a geometric distribution and assumes that year-class survival and recruitment are constant and all ages are equally selected. Geoducks are extremely long-lived, so that the number of animals observed in each 1-y age class is typically low, even for sample sizes in which $n > 1{,}000$. Despite this problem, we chose to preserve the data in 1-y age classes rather than aggregating ages, a procedure that potentially ignores real variability in the original data and may slightly inflate estimates of M (Noakes 1992). It was not possible to estimate site-by-site mortality rates, because no individual site contained enough data to construct reliable catch curves. Age frequencies were therefore pooled from all 14 sites in order to create the catch curve.

To avoid arbitrary choices of the upper and lower ages used in the catch curve "right limb," we established a protocol for data inclusion: The initial upper age limit for the catch curve was the first age at which our sample contained no geoducks (i.e., the first gap in frequency). We then excluded younger age frequencies if they were identified as outliers by Weisberg's (1985) outlier test. Two methods were used to select the lower age limit for the catch curve: (1) The Chi-square procedure described in Robson and Chapman (1961) was used to differentiate partially selected ages, and (2) catch curve regressions were calculated for all possible lower age limits, and we used an *ad hoc* procedure to optimize the coefficient of determination (r^2) and the linearity of positive and negative residuals plotted against age. Once the lower and upper age limits for the catch curve were identified, a Chi-square formula was then used to test goodness of fit of fully selected ages to a geometric distribution (i.e., the Robson and Chapman model). The von Bertalanffy growth parameters estimated at 11 Washington sites from Hoffmann et al. (2000) were used as site-specific growth inputs. Sexual maturity, weight-at-length, and fishery selectivity parameters were derived on the basis of published literature from Washington and British Columbia.

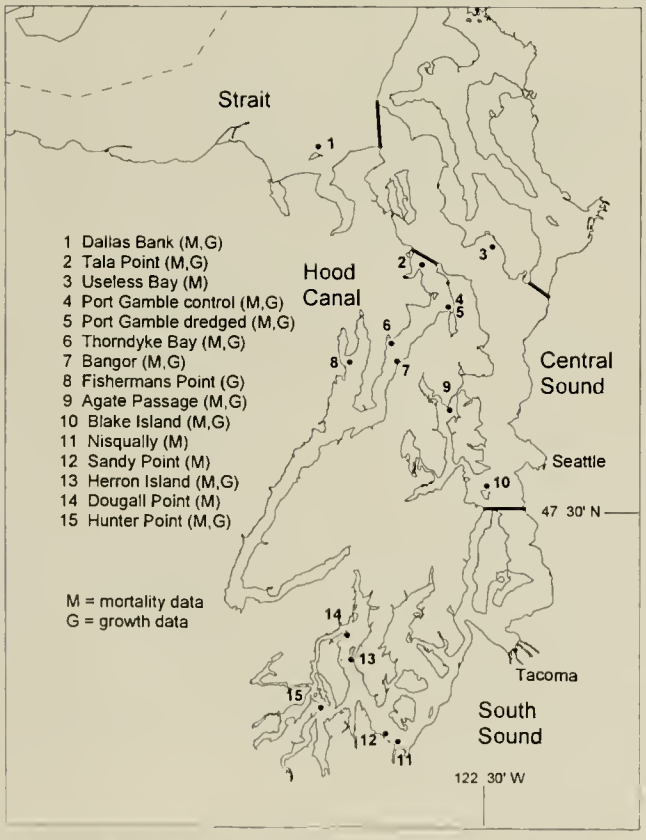

Figure 1. Sampling sites for geoduck natural mortality and growth. Also shown are boundaries for four of Washington's geoduck management regions; the two not shown contained no sampling sites and no surveyed geoduck biomass.

Yield Model

Geoduck yield was modeled using a deterministic, age-structured equilibrium yield model. Given a set of parameter estimates for mortality, maturity, growth, and selectivity, the model collapses the number of geoducks at age for all cohorts in the population to a single cohort, assumed to represent the stable age distribution of the population. Population size was based on an initial unfished spawning population, by a declining exponential function for survival at age, and by the Baranov catch equation (Ricker 1975). Baranov's catch equation says that annual catch is a simple linear function of instantaneous fishing mortality and mean population size. The derivation of Baranov's catch equation is presented in Seber (1982). Seber cites Baranov (1918) as the origin of the catch equation, hence its common name. The model assumed continuous recruitment, the magnitude of which was based on a Beverton-Holt stock-recruitment relationship (Ricker 1975). The Beverton-Holt stock-recruitment relationship, commonly used with marine fish, is an asymptotic function that estimates annual recruitment based on parent stock size. The implication of this relationship is that over a broad range of parent stock size, recruitment is stable, but as parent stocks reach critically low levels, recruitment drops precipitously. A maximum age (a_{max}) in the model served as an "accumulator age" category that encompassed all ages $a \geq a_{max}$. The assumption implicit in this formulation is that no significant changes in growth, weight, maturity, or selectivity occurred beyond a_{max}. In the case of geoducks, this assumption was reasonable and is addressed below. For other applications, the model could be simply extended to accommodate an unlimited number of older age classes. The model was constructed as a QuattroPro for Windows (version 5.0) spreadsheet.

Table 1 lists the user-supplied inputs required by the model. These include estimates of the natural mortality rate, the growth rate, the stock-recruit (S-R) relationship, the unfished spawning biomass, fishery selectivity, sexual maturity, and the population sex ratio. Table 2 shows the parameters derived from the user supplied inputs, listed in computational order. To run the model, fishing mortality (F) was stepped from 0 to a specified upper limit while computing YPR and SPR for each value of F.

The model is capable of returning a suite of fishing mortality

TABLE 1.

Geoduck life history parameter estimates held constant for all study sites.

Parameter Description	Parameter Symbol	Value, Notes
Unfished ("virgin") spawning stock biomass (in kg); the spawning biomass when $F = 0$	$B0_s$	100,000 kg (only required to scale absolute biomass)
Instantaneous natural mortality rate (assumed constant for all ages)	M	0.0226
Weight-at-age (in g) based on length-at-age as derived from the von Bertalanffy growth function	w_a	$w_a = xL_a^y$ L_a = length (cm) at age a x = 0.349127 y = 2.972807
Maturity-at-age; the proportion of female geoducks of age a (in years) that are sexually mature	Φ_a	$\Phi_a = 1/(1 + \exp^{x a+y})$ x = −1.9 y = 9.5
Fishery selectivity-at-age; the proportion of geoducks of age a (in years) selected by the fishery	v_a	$v_a = 1/(1 + \exp^{x a+y})$ x = −1.5 y = 8.0
Beverton-Holt spawner-recruit shape parameter (Kimura 1988)	A	1
Proportion of males in population	p_m	0.5
Maximum (accumulator) age	a_{max}	25

benchmarks, such as F_{max}, $F_{0.1}$, and $F_{xx\%}$. For example, the fishing mortality rate that produces, over the long run, the maximum YPR corresponds to the F_{max} strategy, whereas $F_{0.1}$ represents a rate of harvest less than F_{max} (Deriso 1987, Gulland 1968).

The fraction of the unfished spawning weight per recruit remaining at a given level of fishing mortality was calculated as SPR/SPR0 and is achieved at a corresponding fishing mortality rate $F_{xx\%}$ where xx represents the ratio (SPR/SPR0)100. Model predictions of this fraction formed the basis for SPR-based fishing strategies. For example, the fishing mortality rate that resulted in a value of SPR/SPR0 = 0.35 corresponds to the $F_{35\%}$ strategy.

RESULTS

Natural Mortality

Sampled geoducks from 14 previously unfished sites ranged in age from 2 to 131 y (Fig. 2a). The mean age of geoducks was 46 y (standard error [SE] = 0.56, n = 2,157). The initial upper age limit for the catch curve was 110 y, because no 111-y-old geoducks were in our sample. Examination of residuals showed a single large negative residual at the 99-y age class (only one geoduck of this age was in our sample), and this age class was eliminated from the analysis as an outlier, based on the test given in Weisberg (1985). Both the Robson and Chapman (1961) Chi-square procedure and our *ad hoc* optimization procedure identified age 28 as the lower age limit for the catch curve. A Chi-square was used to test goodness of fit of fully selected ages (28–98) to a geometric distribution. The resulting Chi-square was highly significant (X^2 = 326.56, degrees of freedom = 68), indicating that the age frequency was not geometric in distribution and that data requirements for the Robson and Chapman model were not met. Ricker (1975) pointed out that in most stocks, difference in year-class strength is the major source of variability, in which case the best estimate of survival would be obtained from a catch curve analysis with equal weighting. The Ricker catch curve based on ages 28–98 (Fig. 2b) produced an estimate of M = 0.0226 y^{-1} (±0.0018 SE, n = 71, r^2 = 0.70).

Other Model Parameters

Goodwin (1976) calculated an allometric length-weight relationship for Washington geoducks in log-log form. We converted this to the more familiar power curve form $w_a = xL_a^y$, where w_a = weight (in g) at age a, L_a = shell length (in cm) at age a (Table 1). The proportion of males (p_m) in the geoduck population was set to p_m = 0.5 based on a 50:50 sex ratio for geoducks older than 10 y (Goodwin and Pease 1989).

The proportion of sexually mature geoducks at age (Φ) was estimated by fitting a simple logistic curve to maturity data from published sources. Anderson (1971) found that 50% of his sample of geoducks was mature at 75 mm and an age that he estimated to be 3 y. The Washington growth curves described above suggest that this length would be attained in roughly 5 y, depending on the site. Sloan and Robinson (1984) reported that geoducks mature at 5 y and reproduce for at least a 100-y period with no "reproductive senility." They stated that "unequivocally mature geoducks" were 6–103 y old (late-active males) and 12–95 y old (late-active females). On the basis of these two sources, we fit a logistic curve with the least-squares method and two data points, whereby 50% of the female geoducks would mature at 5 y and 100% by 12 y (Table 1).

The proportion of geoducks at age a selected by the fishery (v_a) was based loosely on Harbo et al. (1983), who reported that recruitment to the British Columbia geoduck fishery begins at 4 y and is complete by 12 y. To more conservatively model fishery selectivity, we fit a simple logistic curve using the least-squares method and two data points, whereby geoducks enter the fishery at roughly 4 y and are fully selected by 8 y (Table 1).

Nothing is known about the form or steepness of the S-R relationship for geoducks. We therefore set the Beverton-Holt shape parameter (A) equal to 1.0 for all model runs. In other words, we assumed that recruitment was independent of spawning stock abundance. This assumption is reviewed below in *Discussion*.

As a practical convenience, the equilibrium yield model uses an "accumulator age" category (a_{max}) as the final age category, encompassing all ages $a \geq a_{max}$. For this study, we set a_{max} = 25, which implicitly assumes that there are no significant changes in growth, selectivity, or maturity beyond age 24. This assumption is reasonable for geoducks, which reach asymptotic size between the ages of 10 and 20 y (Hoffmann et al. 2000).

Fishing Mortality Rates for Five Harvest Strategies

We ran the model for each site, varying only the growth parameters based on the analysis of growth presented in Hoffmann et al. (2000). The only sites where growth parameter estimates (specifically, the growth constant k) could be pooled were five of the six Hood Canal sites. In all other cases, site-specific growth parameters could not be pooled, and therefore separate model outputs

TABLE 2.

Description of derived parameters used in the geoduck equilibrium yield model.

Description	Derived Parameters	Notes
Number of geoducks surviving to the first age class (a = 1 year)	$N_a = p_m$ for males $N_a = 1 - p_m$ for females	p_m = proportion of males in the population (see Table 1)
Instantaneous rate of fishing mortality at age a	$F_a = Fv_a$	F = instantaneous rate of fishing mortality for fully selected age classes (v_a = 1); user-supplied. v_a = fishery selectivity at age a (see Table 1)
Instantaneous rate of total mortality at age a	$Z_a = M_a + F_a$	M_a = instantaneous natural mortality rate (see Table 1)
Annual rate of survival	$S_a = \exp(-Z_a)$	
Number of geoducks surviving to age a for $a > 1$	$N_a = N_{a-1}S_{a-1}$	
Average number of geoducks at age a	$\overline{N_a} = N_a(1 - S_a)/Z_a$ for $a < a_{max}$ $\overline{N_a} = N_a/Z_a$ for $a = a_{max}$	a_{max} = maximum (accumulator) age (see Table 1)
Average biomass (in kg) of geoducks at age a	$\overline{B_a} = \overline{N_a}w_a$	
Yield per recruit (in kg) at age a	$YPR_a = v_aF\overline{B_a} = F_a\overline{B_a}$	
Total yield per recruit (in kg) for all ages	$YPR = \sum_a v_aF\overline{B_a} = F\sum_a v_a\overline{B_a}$	
Spawning weight per recruit (in kg)	$SPR_a = \overline{B_a}\Phi_a$ for age a $SPR = \sum_a \overline{B_a}\Phi_a$ for all ages	Φ_a = proportion of mature females at age a (see Table 1)
Fraction of unfished spawning stock biomass remaining at a given level of fishing mortality	$P = 1 - (1/A)(1 - SPR/SPR0)$	A = Beverton-Holt shape parameter (see Table 1). SPR0 = unfished spawning weight per recruit (total SPR when F = 0)
Spawning biomass (in kg) when $F > 0$	$B_s = P\,B0_s$	$B0_s$ = unfished spawning stock biomass (see Table 1)
Recruitment (in numbers)	$R = (B_s/SPR0)/[1 - A(1 - P)]$	Reference: Kimura (1988)
Yield (in kg)	$Y = YPR(R)$	
Harvest rate for fully selected age classes (v_a = 1)	$\mu = F/Z[1 - \exp(-Z)]$	Reference: Ricker (1975)

were calculated for each site. All inputs except growth parameters were identical for each model run (Table 1). Growth parameters used as site-specific input are shown in Table 3.

Values of the instantaneous fishing mortality rate (F) for five commonly used constant harvest rate strategies are shown in Table 3. F_{max} is the fishing mortality rate that produces, over the long run, the maximum YPR. $F_{0.1}$ is a common alternative to F_{max} and is the rate of fishing mortality at which the marginal YPR is 10% of the marginal YPR for a lightly exploited fishery (Deriso 1987). $F_{35\%}$, $F_{40\%}$, and $F_{50\%}$ are SPR-based harvest rates that reduce SPR to either 35%, 40%, or 50% of the unfished level (Clark 1991).

F_{max} ranged from 0.053 to 0.100 depending on the site (Table 3). These rates correspond to annual harvest rates (μ) of 5.1–9.4% of the exploitable geoduck biomass. The Strait of Juan de Fuca region, represented by the single sampling site at Dallas Bank, produced the lowest value, whereas Fishermans Point in Hood Canal produced the highest value. The F_{max} strategy reduced SPR to 15–21% of the unfished level, depending on the site. Values for $F_{0.1}$ ranged from 0.028 to 0.037, corresponding to annual harvest rates of 2.7–3.6%. This strategy reduced SPR to 35–37% of the unfished level, depending on the site.

Values for $F_{35\%}$ were, predictably, nearly identical to the $F_{0.1}$ rates, ranging from 0.30 to 0.36 (μ = 2.9–3.5%). F values for the $F_{40\%}$ strategy ranged from 0.025–0.030 (μ = 2.4–2.8%), whereas those for the $F_{50\%}$ strategy ranged from 0.018–0.020 (μ = 1.8–2.0%).

Model Sensitivity to Parameter Estimates

All of the parameter estimates used to drive the model are subject to varying degrees of uncertainty. It is therefore reasonable to ask what might happen to our predictions if the true values of M or k, for example, were much lower or higher than our estimates. We tested the sensitivity of the model by running it with a range of values for each parameter in turn while holding all other parameters constant. Values ranging from one-tenth the "best" parameter estimate (from Tables 1 and 2) to three times the estimated value were used in the analysis. Only the fishing mortality rates corresponding to the $F_{40\%}$ strategy were calculated, but the trend for other strategies would be similar.

The model was most sensitive to the estimate of M, with $F_{40\%}$ values ranging from 0.003 to 0.068 as M was increased from one-tenth to three times our "best" estimate of M = 0.0226 (Fig. 3). The model was far less sensitive to the other parameter estimates, as evidenced by the relatively flat $F_{40\%}$ trajectories for values of the growth coefficient k, the selectivity constant γ, and the maturity constant γ. For example, varying the value of k from one-tenth to three times our best estimate resulted in $F_{40\%}$ values that ranged only from 0.021 to 0.033.

Use of Model Results to Set Annual Fishing Quotas

The model results presented above, together with an estimate of exploitable biomass, may be used to set annual fishing quotas. The first step in such a process is for managers to recommend one of the five harvest strategies described above, or an alternate strategy; the model is capable of returning F-values for any desired level of equilibrium spawning biomass or yield. The decision process involved in recommending a particular harvest strategy is by no means clear-cut, but some guidelines on risk-averse strategies from the recent fisheries literature are reviewed below in *Discussion*.

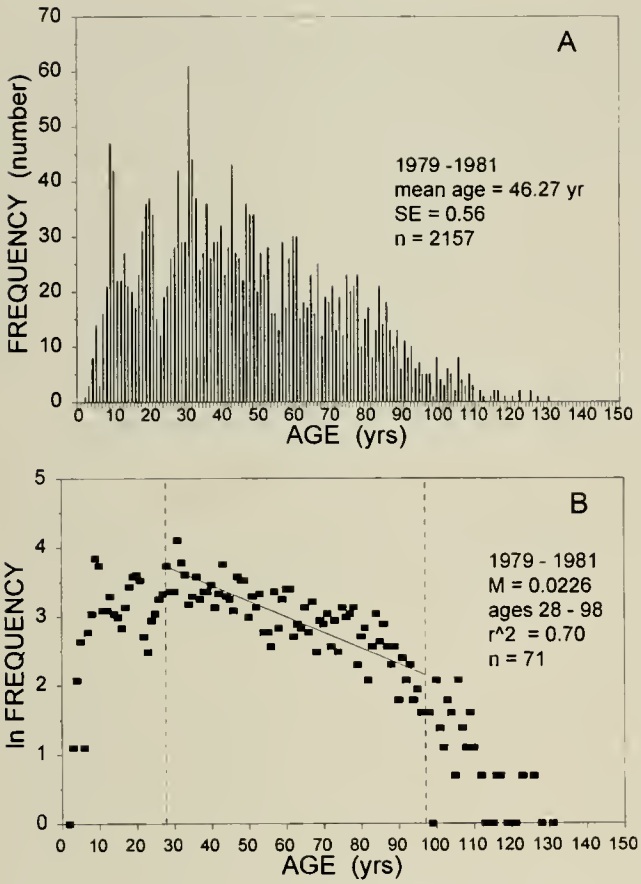

Figure 2. (A) Age frequency of geoducks sampled at 14 sites in Washington. (B) Catch curve used to estimate the instantaneous natural mortality rate (M) of geoducks.

Once managers reach a decision on the "best" harvest strategy, the corresponding F-value may be taken directly from the mean values in Table 3. This mean F-value is then converted to the harvest rate (μ) for fully selected age classes (Ricker's equation from Table 2). To produce the recommended annual fishing quota, the harvest rate is then simply multiplied by the estimate of harvestable biomass. For example, Washington managers have recommended and adopted an $F_{40\%}$ strategy for geoducks in all six management regions. This strategy is achieved with an instantaneous fishing mortality rate of $F = 0.028$ (mean value for all sites, Table 3); the corresponding annual harvest rate for fully selected age classes (μ) is 0.027, or 2.7% of the exploitable biomass. Annual dive survey data provide an estimate of exploitable biomass for each of six management regions. As an example, exploitable geoduck biomass in the Hood Canal Region in 1999 was estimated to be 18,185 t (Sizemore and Ulrich 1999), and the resulting annual quota was (0.027)(18,185 t) = 491 t.

DISCUSSION

Our primary objective in equilibrium modeling was to simulate the long-term results of various geoduck fishing strategies, both in terms of yield and SPR. Before discussing our results, it is perhaps necessary to explain why we attach such importance to geoduck harvest rate strategies, particularly since the differences between many of the modeled options may appear trivial.

In many fisheries, especially those in which biomass is small or estimated with great uncertainty, debating a 1% difference between annual harvest rate options would indeed be trivial. But in Washington's geoduck fishery, where the exploitable biomass is large (73,843 t in 1999; Sizemore and Ulrich 1999) and the price is high, even tiny incremental differences in the recommended harvest rate have tremendous economic significance. Moreover, because geoducks have a low M (and presumably a low intrinsic rate of increase), small differences in annual harvest rates can have profound cumulative effects on stock size, especially if the harvest rate is set too high. This is not to discount the importance of good biomass estimates, but we believe there are several reasons why Washington managers should place the greatest emphasis on improved harvest rate strategies rather than improved biomass estimates. First, biomass estimates for individual geoduck beds in Washington have coefficients of variation (CVs) averaging about 11%. Simulation tests suggest that biomass estimation errors of this magnitude are unlikely to result in substantial degradation of long-term harvest performance (Frederick and Peterman 1995). Second, even greatly increased sampling is not likely to improve biomass estimate CVs very much. Third and most importantly, errors in biomass estimation are assumed to be reasonably unbiased. An error in setting the annual harvest rate, on the other hand, will have a persistent and cumulative effect on stocks in only one direction, either underharvest or overharvest. We therefore believe that, given reasonable estimates of stock size, choosing a harvest strategy remains the most critical aspect of geoduck management.

In this study, we evaluated five common harvest strategies. Our model predicts that fishing at F_{max} will eventually reduce SPR to less than 20% of the unfished level, a threshold below which many fish stocks are assumed to collapse (Thompson 1993). Therefore, F_{max} should be considered a high-risk strategy for geoducks.

Less risky are the SPR-based strategies, three of which were evaluated here. In this study, we assumed that recruitment was independent of stock size at all levels of fishing (Beverton-Holt parameter $A = 1.0$). Although this is the common default assumption in cases in which the S-R relationship is unknown, the risk inherent in this assumption is that given an existing but undetected S/R relationship, $F_{x\%}$ can be greater than F_{MSY} (the preferred fishing rate with a known S/R function; MSY, maximum sustainable yield). As an alternative to F_{max}, SPR-based strategies seek to preserve some minimum level of spawning biomass and at the same time produce yields that are close to the MSY. In an attempt to find fishing strategies that are robust for any likely S-R relationship, recent modeling studies have simulated groundfish yields using a range of typical life history parameters and realistic S-R models. Clark (1991) showed that fishing at $F_{35\%}$ would achieve at least 75% of MSY for a wide range of deterministic S-R relationships. On the basis of his results, $F_{35\%}$ has been adopted as a target rate for a number of fish stocks in Alaska and the U.S. Pacific coast. Clark (1993) later revised his recommendation to $F_{40\%}$ after considering variability in recruitment, but remarked that "it would be silly to argue very hard for or against any specific rate between $F_{35\%}$ and $F_{45\%}$." Mace (1994) also recommended $F_{40\%}$, which she claimed was a modest improvement over $F_{35\%}$. She states that $F_{40\%}$ represents a risk-averse fishing strategy in the common situation in which there is adequate information to place bounds on all relevant life history parameters except the S-R relationship. Quinn and Szarzi (1993) modeled clam fisheries in Alaska and recommended SPR-based strategies equivalent to a range of $F_{30\%}$–$F_{45\%}$.

As noted earlier, Washington managers have adopted an $F_{40\%}$ strategy for geoducks, which corresponds to $F = 0.028$ (averaged

TABLE 3.

Benchmark instantaneous fishing mortality rates for fully selected geoducks (v_a = 1.0) from seven sites in Washington.

Region	Site	n (sites)	L_∞ (cm)	k	t_0	F_{max}	$F_{0.1}$	$F_{35\%}$	$F_{40\%}$	$F_{50\%}$
South Sound	Hunter Point	1	16.4	0.23	0.72	0.090	0.036	0.036	0.029	0.020
	Herron Island	1	13.2	0.15	0.42	0.064	0.031	0.032	0.027	0.018
Central Sound	Agate Passage	1	15.8	0.20	0.18	0.085	0.035	0.035	0.029	0.020
	Blake Island	1	14.6	0.16	0.81	0.064	0.031	0.032	0.027	0.019
Hood Canal	Five sites pooled	5	12.8	0.16	0.47	0.067	0.032	0.033	0.027	0.019
	Fishermans Point	1	16.8	0.24	0.55	0.100	0.037	0.036	0.030	0.020
Strait	Dallas Bank	1	12.0	0.11	0.33	0.053	0.028	0.030	0.025	0.018
Mean of all sites						0.075	0.033	0.033	0.028	0.019

Model inputs except growth parameters are from Table 1. Growth parameter estimates are from Hoffmann et al. (2000).

over all sites) and annual harvest rate (μ) of 2.7% of current exploitable biomass. British Columbia managers calculate annual quotas using a fixed harvest rate of 1% (Campbell et al. 1998), but this rate is applied to the estimated *virgin* biomass rather than current biomass estimates, as is done in Washington.

A secondary objective of our study was to determine which of the estimated geoduck life history parameters were most influential in predictions of yield and SPR. The model was most sensitive to the estimate of natural mortality (M), whereas growth, selectivity, and maturity parameters had relatively little effect on SPR-based fishing mortality rates. This suggests that future research monies are best spent making more reliable estimates of M.

Because our model is an equilibrium model and admittedly sensitive to the estimate of M, one could ask how it might cope with time varying natural mortality. If it were possible to construct a functional relationship between specific, measurable categorical variables—such as predator density, or sea temperature and natural mortality rates—and if these categorical variables were themselves predictable, one could estimate the expected changes in M. With a credible estimator, the equilibrium model could be converted to a dynamic pool model and revised estimates of F could be derived for a specific future time interval of interest. Such an application would be highly dependent on the accuracy and precision of the predictive functions, not only the functions related to M but also the expected annual recruitment. We are doubtful that this approach would become profitable. Alternatively, annual or fixed interval updates of the equilibrium F could be computed using revised estimates of M.

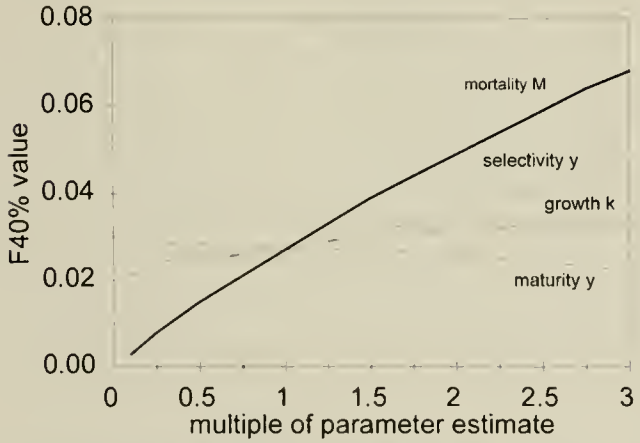

Figure 3. The effect of different parameter estimates on model-derived $F_{40\%}$ values. Numbers on the X-axis represent multiples of the "best" parameter estimates from Table 1 (mortality, selectivity, and maturity) and Table 3 (growth parameter k).

If natural mortality varies over time, the true $F_{40\%}$ would rise and fall proportionately with the change in M (Fig. 3). We would err in the application of our equilibrium F dependent on the trend in M. If M fluctuates around some normally distributed mean, then on average our equilibrium F is probably reasonable. If there is a significant periodicity in the trend in M (a long duration decline, for example) and it goes unrecognized, application of the equilibrium F risks overharvest of the resource. Managers could impose a safety valve by creating a harvest policy that reduces the exploitation rate below that derived from the preferred F (e.g., 0.75 F), but it would be speculative whether this precaution was sufficient to account for real variability in M. Models of stochastic variability in recruitment have led scientists to suggest maintaining a larger spawning biomass and therefore adoption of a lower preferred F (e.g., $F_{40\%}$ rather than $F_{35\%}$) (Mace 1994, Clark 1993).

Our estimate of M = 0.0226 is similar to estimates from British Columbia. Sloan and Robinson (1984) estimated M = 0.035 at a single site, while Breen and Shields (1983) reported M = 0.01–0.04 in five populations. Noakes (1992) estimated M = 0.03–0.04 at three sites. Both our estimate and the British Columbia estimates relied on the catch curve method, which assumes that mortality rate is uniform with age and that recruitment has been constant over the range of age groups analyzed. There is some suggestion in our age-frequency data that a shift in geoduck recruitment has occurred that could have biased the estimate of M. Age frequencies did not begin to decline until about age 25, a pattern in catch curves that is often due to inefficient sampling of younger age classes. But for geoducks, which grow quickly and are fully selected by the commercial fishery at half this age (Harbo et al. 1983), sampling inefficiency is not a plausible explanation for the low numbers of geoducks in the 10–25-y age group. Instead, low numbers of 10–25-y-old geoducks may indicate poor recruitment during the 15-y period before sampling. This suggests that recruitment declined during the period 1955–1970 (before the advent of a fishery) and perhaps more recently. Sloan and Robinson (1984) suggested the possibility of a similar decline in recruitment during the same time period in British Columbia.

Thus, catch curve estimates of M for geoducks based on older age classes may not accurately represent current trends in natural mortality. They likewise reveal nothing about M for younger geoducks. In either case, our results indicate that biases in the estimate

of M will have a major influence on model-based predictions of yield and SPR. Independent estimates of M should therefore be a high priority for research. Given the fact that geoducks are entirely sedentary, direct or "known fate" estimates of M may be possible if a reliable and noninvasive tag can be developed. Such straightforward measurements of annual mortality would rely on fewer assumptions than the catch curve method and might also provide age-specific and area-specific estimates M.

A final caveat related to the use of simple yield models such as ours is that they do not take into account the spatial distribution of harvested animals. Spatial structure is frequently ignored in the management of finfish stocks, because it is assumed that survivors are being continually mixed by movement. Under this "dynamic pool" assumption, it does not matter whether the annual quota is taken in small amounts over the entire fishing area or taken entirely within a tiny corner of that area. But as Orensanz and Jamieson (1998) point out, the dynamic pool assumption may be risky when applied to sedentary benthic species such as geoducks. More research should therefore be devoted to the long-term effects of various spatial harvesting strategies on yield and spawning biomass of geoducks. An experiment of this sort is underway in Washington, where geoduck densities at 15 commercial beds are being monitored before and after fishing to estimate an empirical rate of population recovery. If it is based on a long span of time, an empirically determined turnover (i.e., recruitment) rate for commercially fished geoduck beds could be used to validate, improve, or replace the harvest rate strategies on the basis of structural models.

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MICROSPORIDIOSIS IN QUEEN SCALLOPS (*AEQUIPECTEN OPERCULARIS* L.) FROM U.K. WATERS

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ABSTRACT Spores of a microsporidian parasite were found in the queen scallop, *Aequipecten opercularis* (L.), collected from several coastal sites around the United Kingdom. Developing spore stages were detected in contact with the host cell cytoplasm. Infected host cells formed aggregates in the connective tissue of the digestive gland. Fully mature spores were found free within blood vessels. These spores exhibited a dome-shaped polaroplast, a diplokaryon, a posterior vacuole, and an isofilar polar tube, with seven to eight coils in a single row. In immature spores, the number of coils in the polar tube varied, with some having 7 to 8 coils and others having 10 to 12 coils.

KEY WORDS: Microsporidian, spores, scallop, *Aequipecten opercularis*, parasite

INTRODUCTION

Scallop species are of commercial interest in many parts of the world. In Europe, the main species exploited is the great scallop (*Pecten maximus*), which is the subject of important fisheries in France, Ireland, the United Kingdom, and Norway, where it is cultivated on a small scale. There is also a significant natural fishery for the queen scallop, *Aequipecten opercularis*. This species is distributed from northern Norway and the Faroe Islands to the Mediterranean and Adriatic Seas. It lives in depths ranging between 18 and 46 m on fine sand, fine gravel, or sandy gravel. The main areas of fisheries for this species lie along the western coasts of the British Isles and France but also include the Shetland Isles and Moray Firth in the northeast and both sides of the English Channel (Ansell et al. 1991, Brand 1991).

Since little is known on the natural disease status in these two species, a survey was undertaken to collect baseline data on parasites and possible pathogens in natural, apparently healthy populations. During this study, a microsporidian was found in the digestive gland of the queen scallop, and electron microscopy (EM) studies were undertaken for characterization and identification of this parasite.

Microsporidians are eukaryotic, obligate intracellular parasites of almost all animal phyla. The most common hosts are arthropods and fish (Canning 1990). Only a few microsporidians have been reported from bivalves. Comps et al. (1975) found an unidentified species in *Cardium edule* and Jones (1981) described *Microsporidium rapua* from the oyster *Ostrea lutaria* in New Zealand. In mytilids, a microsporidian parasitizing the oocytes has been described by Figueras et al. (1991a, 1991b) who found *Steinhausia mytilovum* in *Mytilus galloprovincialis* from Spain and in *Mytilus edulis* from the United States. Villalba et al. (1997) observed the same parasite in ova of *Mytilus galloprovincialis*, and Sagristá et al. (1998) described the developmental cycle and ultrastructure of this protistan in *M. galloprovincialis*.

In this study, the spores of a microsporidian parasite from the queen scallop are described.

MATERIALS AND METHODS

A total of 454 adult queen scallops (*A. opercularis*) were sampled during the period July 1997 to April 1998. The sampling localities are shown in Figure 1. The localities and dates of sampling are shown in Table 1.

For histology, transverse tissue sections were taken that included the digestive gland, kidney, gills, gonad and mantle, and they were fixed in Davidson's fixative (Shaw and Battle 1957) for 24 h. The tissues then were dehydrated, cleared, infiltrated with paraffin wax, and sectioned at 5–6 μm. The routine stain used for all samples was Gill's hematoxylin and aqueous eosin. Selected samples were stained with special stains: Farley-Feulgen (Farley 1969) for identifying DNA, and Giemsa for staining presumptive parasites. For each sample, one section was cut, and the slides were examined using a Reichert Polyvar microscope. Photographs were taken with a photomicroscope (E800 Eclipse, Nikon, Tokyo, Japan).

For transmission EM, 1-mm^3 pieces of digestive gland from each specimen sampled for histology were fixed in 3% glutaraldehyde in 0.2M cacodylate buffer with 1.75% NaCl, for 2 h at room temperature, and were washed in the same buffer. After histological assessment, those samples found to harbor the microsporidian were further processed for EM. Tissues were washed another three times in 0.2M cacodylate buffer with 1.75% NaCl, and were postfixed for 1 h in 1% osmium tetroxide in the same buffer. After washing twice with buffer, they were rinsed in distilled water, stained for 1 h *en bloc* with 2% aqueous uranyl acetate, dehydrated in ethanol, washed in propylene oxide, and embedded in Epon 812 (premix, BDH). Semithin sections, 1 μm thick, were cut on a Reichert Ultracut S microtome and were stained with toluidine blue. Ultrathin sections were cut with a diamond knife and were stained with aqueous uranyl acetate and lead citrate. The sections were viewed and photographed with an electron microscope (EM 900, Zeiss) at 50 kV.

Measurements from stained histological or semithin sections were made using a Nikon E800 microscope with LUCIA screen

Figure 1. Map of England and Wales showing the sampling sites.

measurement system. The number of longitudinally sectioned spores that were measured is indicated in each case.

RESULTS

The prevalence of microsporidiosis in *A. opercularis* is given in Table 2.

Spores were found in two different locations in the digestive gland of *A. opercularis*. Immature spores were found in the cytoplasm of connective tissue cells, and mature spores were observed free in blood vessels. In some scallops, both kinds of spores were seen together in the same section; in others, only one kind of spore was found (Fig. 2).

The cells with maturing spores formed aggregates measuring approximately 300 μm in diameter. They were found in two of the 80 resin blocks examined. The whole aggregate (Fig. 2) as well as each infected host cell was surrounded by layers of fibroblast-like cells, as shown at the EM level (Fig. 3). In infected cells, the nucleus could be observed in some sections. The cytoplasm generally was degraded, although mitochondria could still be recognized. The presence of a sporophorous vesicle was not confirmed, the spores being in direct contact with the host cytoplasm. The spores measured 2.3 (range 1.8–2.8) × 1.3 (range 1.1–1.9) μm (n = 20), had an elongate-ovoid shape, and showed different degrees

TABLE 1.

Localities and dates of sampling.

Locality Name	Site Reference	Number Sampled	Date of Sampling
Isle of Man, Bradda Offshore	Site A	5	05/08/97
Isle of Man, Douglas	Site A	150	18/11/97
Red Wharf Bay	Site B	59	16/01/98
West of Portland Bill	Site C	33	24/09/97
West of Portland Bill	Site C	117	14/10/97
West of Portland Bill	Site C	50	24/04/98
Humber Rough	Site D	40	08/01/98

TABLE 2.

Prevalence of microsporidiosis in *A. opercularis*.

Isle of Man (A)	Red Wharf Bay (B)	Portland Bill (C)	Humber Rough (D)
4.5%	10.2%	12.5%	20%

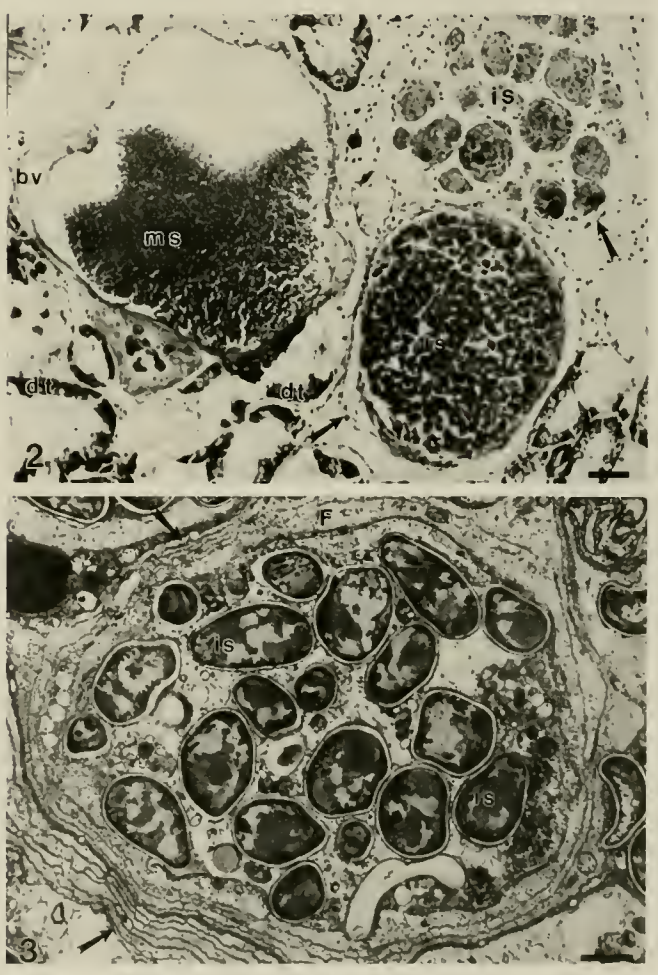

Figure 2. Histological section showing mature spores (ms), free in a blood vessel (bv), and two aggregates of host cells containing immature spores (is). The upper aggregate is less mature, and both aggregates are surrounded by fibroblast-like cells of host origin (arrows); dt = digestive gland tubule (Giemsa stain; bar = 50 μm).

Figure 3. Transmission electron micrograph of one infected cell with immature spores (is), surrounded by fibroblast-like cells of host origin (F). The cell membranes of these cells form layers around each infected cell (arrows) (bar = 1 μm).

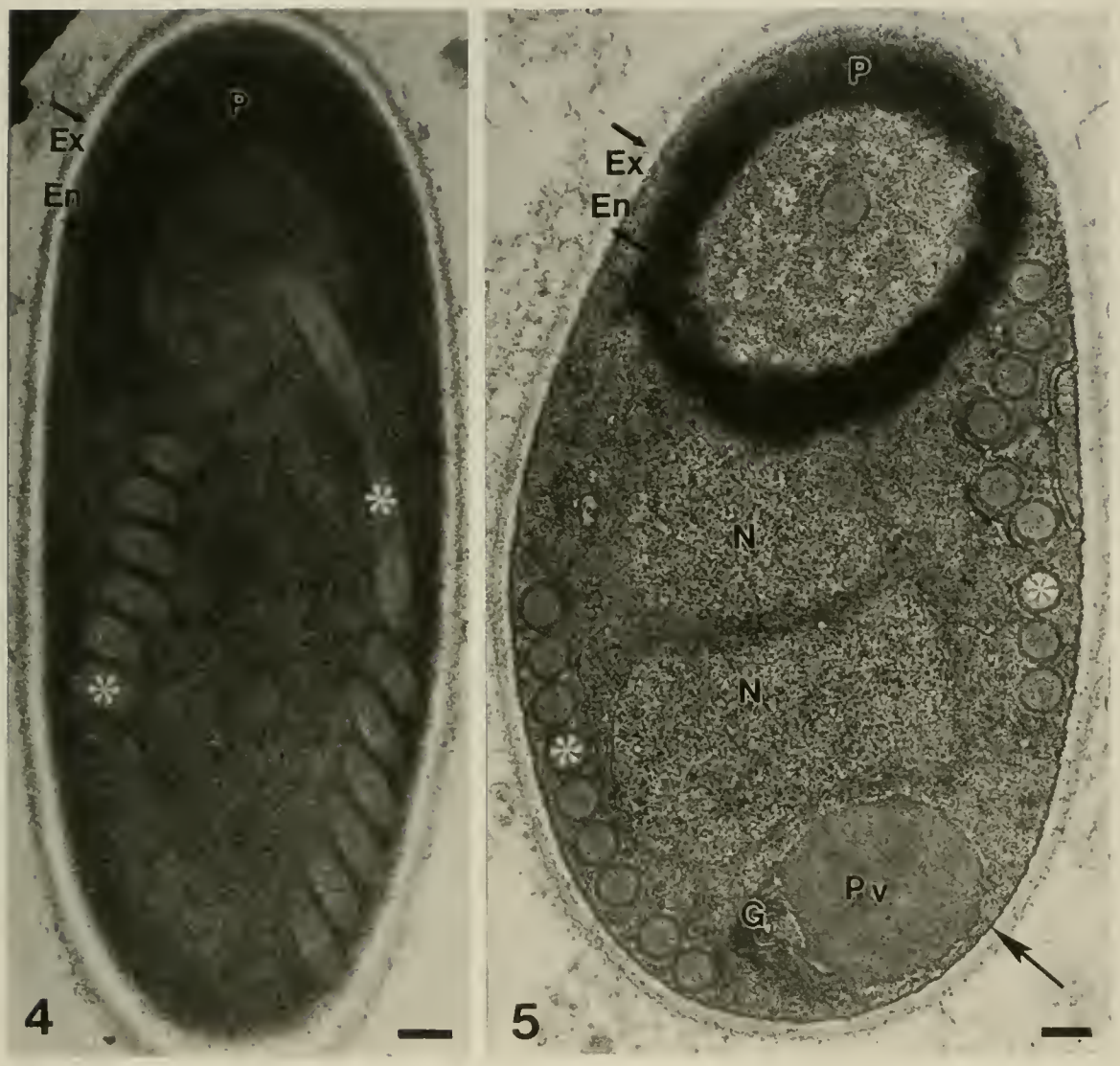

Figure 4. Mature spore viewed in longitudinal section. The exospore (Ex) and the endospore (En) can be observed. Internally, the polaroplast (P) and oblique sections of the polar tube can be seen (*) (bar = 0.1 μm).

Figure 5. Electron micrograph of a slightly oblique section of a mature spore. The outer covering consists of an exospore (Ex), endospore (En), and the cell membrane (arrow). The polaroplast can be observed at the anterior end of the spore, with the two nuclei (N) forming a diplokaryon in the central region of the spore. The coils of the polar tube in transverse section (*), the posterior vacuole (Pv), and in close proximity Golgi-like membranes (G) can also be seen (bar = 0.1 μm).

of maturation. It was not possible to determine whether the immature spore contained a single nucleus or a diplokaryon, since areas considered to be nuclear did not appear to be delimited by an envelope. The polar tube was isofilar, with 10 to 12 coils in a single row. Some spores showed a shorter polar tube, with seven to eight coils. The spores were limited by an inner electron-lucent endospore and an outer electron-dense exospore.

Mature spores were found in only one of the resin blocks examined. They were located in blood vessels and were elongate-ovoid in shape, measuring 2.3 (range 1.9–3.2) × 1.2 (range 0.8–1.7) μm (n = 9). They were limited by an outer exospore and an electron-lucent endospore covering the plasma membrane (Figs. 4, 5). The polar tube was inserted into the anterior anchoring disc (Fig. 6), passing through the center of the spore, and then, in the posterior two thirds of the spore, were wound in most cases into 7 to 8 coils (Fig. 7), and exceptionally into 9 coils (Fig. 5), and were aligned in a single row. The polar tube was isofilar, measuring 83.5 nm in diameter. A conspicuous, dome-shaped polaroplast occupied the anterior third of the spore, enclosing the straight region of the polar tube and terminating close to the coiled polar tube (Fig. 4). Two spherical nuclei were closely apposed, forming a diplokaryon. Each measured up to 0.88 μm in its longest axis and was flattened in the zone of contact with the other nucleus (Fig. 7). The diplokaryon was located in the central third of the spore, between the polaroplast and the posterior vacuole. The latter was limited by a single membrane, with Golgi-like membranes often present in close association (Fig. 5).

DISCUSSION

This is the first time that a microsporidian infection has been reported in any scallop species.

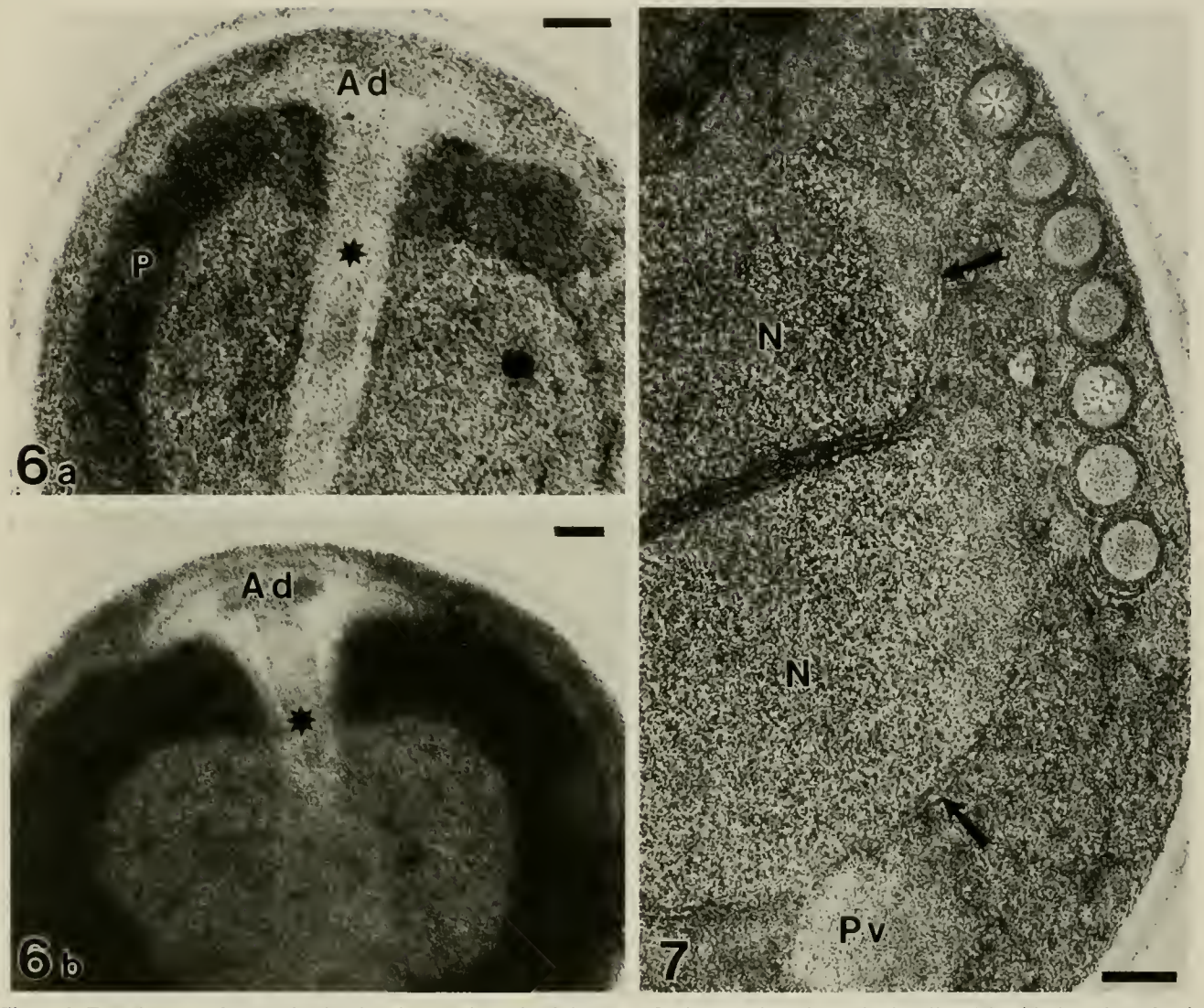

Figure 6. Two electron micrographs showing the anterior pole of the spore. In these sections the anchoring disc (Ad) with the polar tube (*) attached can he seen. The polaroplast (P) is also apparent (bar = 50 nm).

Figure 7. Electron micrograph from a spore sectioned through the nuclei (N) of the diplokaryon. The nuclear envelope can he clearly observed (arrows). The polar tube (*) has seven coils, and the posterior vacuole (Pv) is also present in this section (bar = 0.5 μm).

Spores were found in two different locations in the digestive gland. Immature spores were located intracellularly, and mature spores were located within blood vessels.

We were unable to find any of the earlier stages of this parasite in the scallops examined. Unlike in other microsporidian species, where developmental stages and spores are present concurrently (Comps et al. 1979, Amigó et al. 1996, Johnson et al. 1997, Larsson et al. 1997), in this species only one developmental stage, the spore, could be observed. It is possible that the early stages were present in a tissue other than that of the digestive gland. Since the cells infected by the microsporidian appear to be hemocytes, the potential for infection in tissues apart from the digestive gland, such as the intestine, stomach, and gills should be recognized. Additional EM studies are needed to investigate this possibility. That the microsporidian has an intermediate host within which the early developmental stages could be present should also be considered. Although most microsporidians have only one host, there are several examples of the requirement for an intermediate host (i.e., *Amblyospora*) (Andreadis 1985, Becnel 1992).

The immature spores differed slightly in the length of the polar tube, some having 7 to 8 coils, and others showing 10 to 12. The presence of two types of spores differing mainly in the length of the polar tube has been described for other microsporidian species, i.e., *Nosema* spp. (Iwano and Ishihara 1991) and *Nosema muscidifuracis* (Becnel and Geden 1994). These authors suggest that a shorter polar tube is characteristic of spores involved in infection of other cells in the same host, the longer polar tube belonging to spores that are involved in transmission from host to host. All the mature spores examined in the present study had a short polar tube, but they were all from one specimen and from the same resin block. We presume that mature spores with a long polar tube also exist, because we found them in the immature spores. Despite the large number of resin blocks examined, we did not succeed in finding the mature spores with 10 to 12 coils of the polar tube.

Fully mature spores showed clear evidence of a diplokaryon. This feature, together with a polar tube consisting of eight coils in a single row, the overall dimensions, and the fact that it is infecting an invertebrate host, places this microsporidian near to the genus

Pseudopleistophora (Sprague et al. 1992), a microsporidian first described as *Pleistophora* sp. parasitizing eggs of the annelid *Armandia brevis* by Szollosi (1971).

One important point that needs to be explained is the mechanism by which immature spores contained in individual cells later appear as free, mature spores in blood vessels. No transitional forms were seen in the current study, but in some reports, as the maturation of the spores progresses, the host cells start to loose their plasma membranes and become a syncytium (Weiser 1976). In this way, the spores would be released to reinfect adjacent cells or to become phagocytosed and perhaps migrate to other tissues.

No host reaction against this microsporidian was seen, other than a thin capsule made by fibroblast-like cells. This protistan does not seem to be a threat to queen scallops, as those sampled showed no evidence of poor condition. However, if these scallops become stressed due to changes in temperature, salinity, or crowding, as occurs in culture situations, the parasite could potentially become harmful to the host (Sindermann 1990). Despite the high prevalence of microsporidiosis in animals from a variety of locations around the United Kingdom, the impact of this parasite on wild populations of *A. opercularis* remains unknown. Further studies are needed to investigate the pathogenicity of the microsporidian in *A. opercularis* held in laboratory conditions under different temperatures and stocking densities. In addition, the identification of potential intermediate hosts and early developmental stages of the parasite are required for a specific identification of this microsporidian.

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EVALUATION OF THREE METHODS OF BOTTOM CULTURE OF THE TROPICAL SCALLOP *EUVOLA (PECTEN) ZICZAC* (L. 1758)

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ABSTRACT Three methods were used to study the growth and survival of juvenile *Euvola ziczac* (initial shell height of 40.4 mm SD = 4.2), and initial dry mass tissues of 0.35 g (SD = 0.01), which were set out at a density of 15 individuals m^{-2} on a sandy bottom at Turpialito in the Golfo de Cariaco, Venezuela. The first method was applied on an area of 3 × 5 m (15 m^2) with minimum demarkation (0.20-m low walls) on the bottom, the second method was applied on 1 × 1 m corrals with 1-m high walls, and the third method on 1 × 1 × 1 m cages with bottom and top covers. Both treatments with high walls were conducted with 15 replicates. We could not quantify growth and survival in the first treatment, because the rate of escape was >80% $month^{-1}$ (12 scallop m^{-2} $month^{-1}$). In the corrals, the escape rate increased progressively from 4% (1 scallop m^{-2} $month^{-1}$) to 36% (5 scallop m^{-2} $month^{-1}$), suggesting that the swimming ability of *Euvola ziczac* increased with size from an initial 40.4 mm to final 69.7 mm in shell height obtained in this study. No scallops escaped from the cages, but survival was less than in the corrals. Our observations suggest that the most appropriate bottom culture method would be corrals with walls higher than 1 m.

KEY WORDS: *Euvola ziczac*, bottom culture, scallop, enclosure, grow-out

INTRODUCTION

Euvola ziczac is a functional hermaphrodite scallop present from Cape Hatteras, North Carolina, throughout the Gulf of Mexico and the Caribbean Sea to southern Brazil off Santa Catarina (Abbott 1974). Although *Euvola ziczac* does not form dense natural banks able to support commercial fisheries activity, the species is considered to have great potential for commercial aquaculture activity off the Bermudas, Columbia, Venezuela, and Brazil (Hernández 1990, Vélez and Lodeiros 1990, Waller 1991, Castellanos et al. 1997). In Venezuela, several studies have determined aspects of biological feasibility for culture in the marine environment under hanging culture conditions (Freites et al. 1993, Freites et al. 1995, Freites et al. 1996, Lodeiros and Himmelman 1994). In this manner, rapid growth (up to 30–35 mm) and high survival rate have been attained. However, in larger sizes, diverse factors intrinsic to suspended culture, such as fouling (Lodeiros and Himmelman 1996), wave action (Freites et al. 1999), and food quality (Hunaulth et al. unpublished data), linked with unfavorable periods of high temperature, low available food, and reproduction effort in this species, generating stressful conditions, which lead to a decrease in growth and survival, have been noted (Lodeiros and Himmelman 1994, Lodeiros and Himmelman 2000). However, when *Euvola ziczac* is cultured in contact with the sandy substratum on the seabed (its natural habitat), high growth and survival rates have been noted, considering bottom culture as the most appropriate for the grow-out stage of the species (Vélez et al. 1995, Hunaulth et al. unpublished data).

Studies of the feasibility of various bottom culture techniques have been made for numerous pectinid species including *Chlamys farreri* (Wang et al. 1992), *Placopecten magellanicus* (Kleiman et al. 1996), *Pecten maximus* (Cliche et al. 1994, Dao et al. 1995), *Patynopecten yessoensis* (Aoyama 1989, Ito 1991), *Argopecten circularis* (Caceres-Martínez et al. 1986; Maeda-Martínez et al. in press), and *Pecten novaezelandiae* (Bull 1991). So, bottom culture is an alternative that has shown important levels of profitability in other scallop species. This is because of a lower investment in equipment, consumables, and maintenance than with the hanging method (Frishmand et al. 1980, Felix-Pico et al. 1991, Gilbert and Leblanc 1991, Wang et al. 1992, Kleinman et al. 1996).

In this manner, the aim of this study was to evaluate the growth and survival of scallop *Euvola ziczac* applying two bottom culture grow-out methods: with barriers in the cage and corral enclosures and with no barriers, to obtain market size.

MATERIALS AND METHODS

This study was conducted over a 6 month period (February 27–September 7, 1994) off the south coast of the Golfo de Cariaco, eastern area of Venezuela (Fig. 1). The individuals used in the experiment were obtained from a hatchery under controlled conditions at the end of August 1993, following the methodology described by Vélez and Freites (1993). Scallops were held in suspension for intermediate culture following the methodology described by Freites et al. (1993, Freites et al. 1995) until the initial mean shell height for the study of 40.4 mm (SD = 4.20) and initial dry mass tissues of 0.35 g (SD = 0.01) was obtained. A total of 720 individuals of *Euvola ziczac* were divided into three batches of 240 individuals each and thereafter, we took 15 individuals for each batch to the initial sample. Later, the remaining 225 individuals of each batch were allotted to the cages, corrals, and the barrier-free method. In the case of the enclosures, 15 replicates were introduced, 12 of which were experimental and three replacements. The latter were introduced to maintain density of the individuals reduced by the effects of mortality and escape. In the case of the barrier-free method, a total area of 15 m^2 was evaluated.

The cages measured 1 × 1 × 1 m, built with galvanized iron bars 8 mm in diameter, lined on the six sides by a galvanized wire mesh

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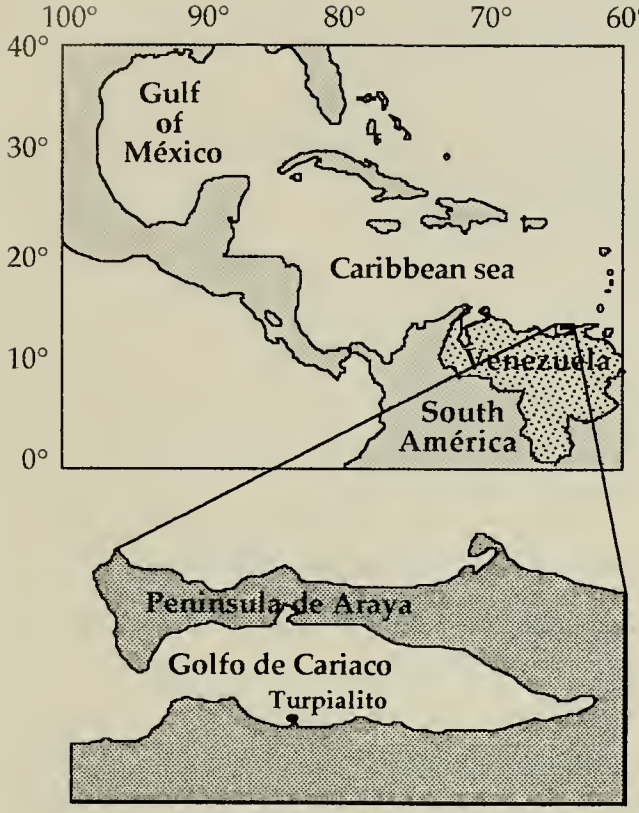

Figure 1. Geographical location of the study area.

with a 30-mm diameter opening (Fig. 2a). The corrals were built of the same size and with the same materials as the cages, except that the galvanized mesh was not fitted on the top and bottom parts (Fig. 2b). Both types of enclosures were buried 7–8 cm into the sand to allow the scallops in cages also to bury, and in case of the corrals, to avoid the escape of individuals under the enclosure and at the same time, to avoid entry of such predators as gastropods and crabs. The individuals in the barrier-free method were distributed in the area marked out beforehand by galvanized mesh, but with an edge of 20 cm. This was used to mark out the original area and thus enabling control of the density but not to act as a barrier (Fig. 2c). Both cage and corral methods were randomly placed at a depth of 7–8 m by a SCUBA diver. Density was a common parameter (15 individuals m^{-2}) both for the enclosures and for the barrier-free method.

Growth of individuals in the enclosures was followed by sampling the three replicates of five individuals (15 individuals) taken randomly, by previously allotting them random numbers. These samples were obtained over approximately 60 days. Also, the number of dead and live individuals was quantified monthly in all the experimental replicates in terms of determining mortality, escape, and monitoring the density of individuals.

The parameters for evaluating growth were shell height (distance between the anterior–posterior margins taken with a Vernier calliper with 0.01 accuracy) and the dry mass of the shell, gonad, muscle, digestive gland, and remaining somatic tissues (dried at 80 °C for 72 h).

Because there was an initial escape rate on the order of 84% of the individuals placed in the original area with the barrier-free method and because these could not be recovered, the evaluation of this method could not continue. Moreover, because of scallop in corrals escaping, we could not continue the evaluation of methods for longer than the 6 months of the study. To evaluate the results on the enclosures, cages, and corrals during the experimental period, the paired student's t test was applied to all growth parameters. Also, in terms of evaluating the masses and heights attained at the end of the study, the nonpaired student's t test was applied. To evaluate the survival rate, because the data were incompatible with assumed normal levels, analysis was conducted by nonparametric tests not correlative to those previously noted (Wilcoxon and Mann-Whitney range tests, respectively, following the recomendations in Zar (1984). For all test a $\alpha = 0.05$ was applied.

RESULTS

Escape

At the start of the experiment, an 84% escape rate was found (12 scallops m^{-2} $month^{-1}$) from the original area using the barrier-free method (Table 1). Furthermore, despite having searched an approximate area of 2500 m^2 taking the original area as the center, none of the individuals (0% recovery) was recovered, so that we were unable to continue with the evaluation. In the corrals, a progressive increase in monthly escapes was noted, from 4% (1 scallop m^{-2} $month^{-1}$) rising to 36% (5 scallops m^{-2} $month^{-1}$), observed at the end of the experiment (Table 1). In this way, the ratio of growth in shell height with the percentage increase in escape of individuals reared in cage was directly proportional ($P < 0.05$, $r^2 = 0.89$; $b = 2.15$). No scallops escaped from the cages.

Survival Rate

Monthly survival rates in the two enclosures showed similar trends (Fig. 3a) (Wilcoxon test, $P = 0.679$). At the end of the study, however, accumulated mortalities result in a significantly lower survival rate of the individuals in cages (51%); whereas, in the corrals, it was in the order of 78% (Fig. 3b) (Mann-Whitney test, $P < 0.05$).

Shell Size and Mass

Growth curve trends in shell height, both for individuals in cages and in corrals, were similar throughout the study period (paired student's t-test, $P = 0.912$), with the exception of the last sampling, where a reduction in the growth rate of cage-reared individuals was observed (Fig. 4a). At the end of the experimental period, the individuals reared in corrals had an average of 73.1 ± 2.34 mm; whereas, the average for the cages was 69.7 ± 3.93 mm. These differences, however, were not significant (nonpaired student's t-test, $P = 0.082$). The dry mass of the shell showed a growth pattern similar to that of shell length during almost the entire study period (Fig. 4a, b), but in this case, significant differences were noted (paired student's t-test, $P < 0.05$). So, there were significant differences (nonpaired student's t-test, $P < 0.05$) noted in the shell growth rates at the end of the study between scallops in corral (26.5 ± 2.61 g) compared to the individuals maintained in cages (23.9 ± 3.72 g).

Somatic Tissue Mass

The growth trend for somatic tissues muscle, digestive gland, and the remaining somatic tissues observed during the study period in cages and corrals, (Fig. 4c, d, e), showed no significant differences (paired student's t-test, $P = 0.719$, 0.679, and 0.369, respectively), despite the fact that these showed divergences in the

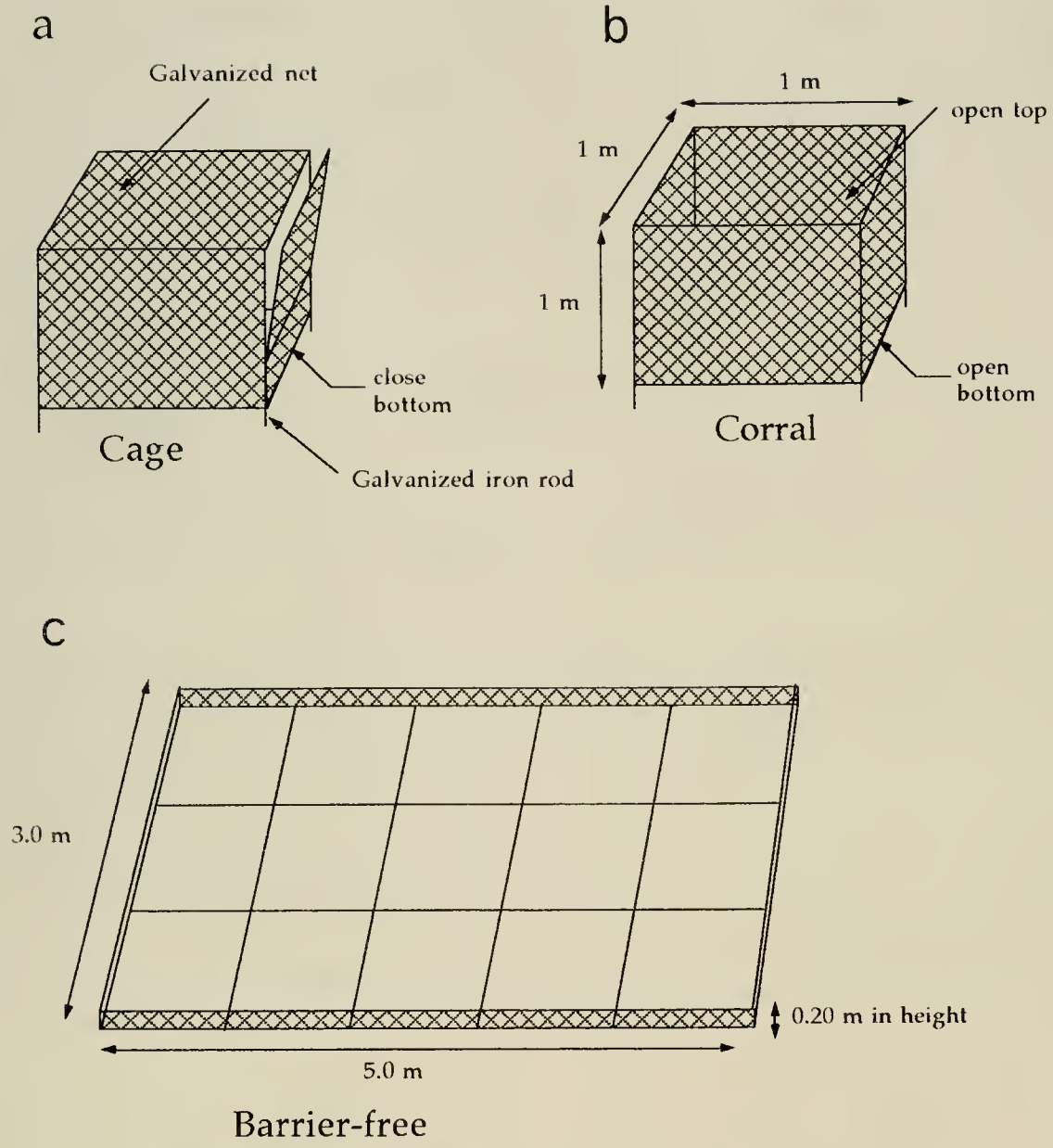

Figure 2. Design of enclosure cages (a) and corrals (b) and the barrier-free method (c).

latter period of the sampling, particularly in the remaining somatic tissues of both groups of individuals. Also, the decrease in growth of the mass of remaining tissues and gonads of individuals maintained in cages, observed at the end of the experimental period, contrasted with the increase in mass of these tissues in the corral-reared individuals (Fig. 4d, f), in such a manner that these were significantly greater at the end of the experimental period (nonpaired student's t-test, $P < 0.05$). In the case of the muscle mass in individuals reared in cages and corrals, no significant differences were shown at the end of the study period (nonpaired student's t-test, $P = 0.947$).

DISCUSSION

Our results showed that by applying the bottom culture method, in the course of approximately 1 year (from fertilization of the oocytes), sizes and biomasses considered as commercially feasible

TABLE 1.

Monthly escape (%) of the scallop *Euvola ziczac* observed during the study in the methods evaluated: barrier-free and cage.

Month	Methods	Shell Height	% Escape
April	Barrier-free*		84
	Corrals	54 mm	4
May	Corrals	59 mm	10
June	Corrals	65 mm	18
July	Corrals	67 mm	33
September	Corrals	70 mm	36

In the cages, the percentage of escape was always of 0%.
* We were unable to continue with the evaluation.

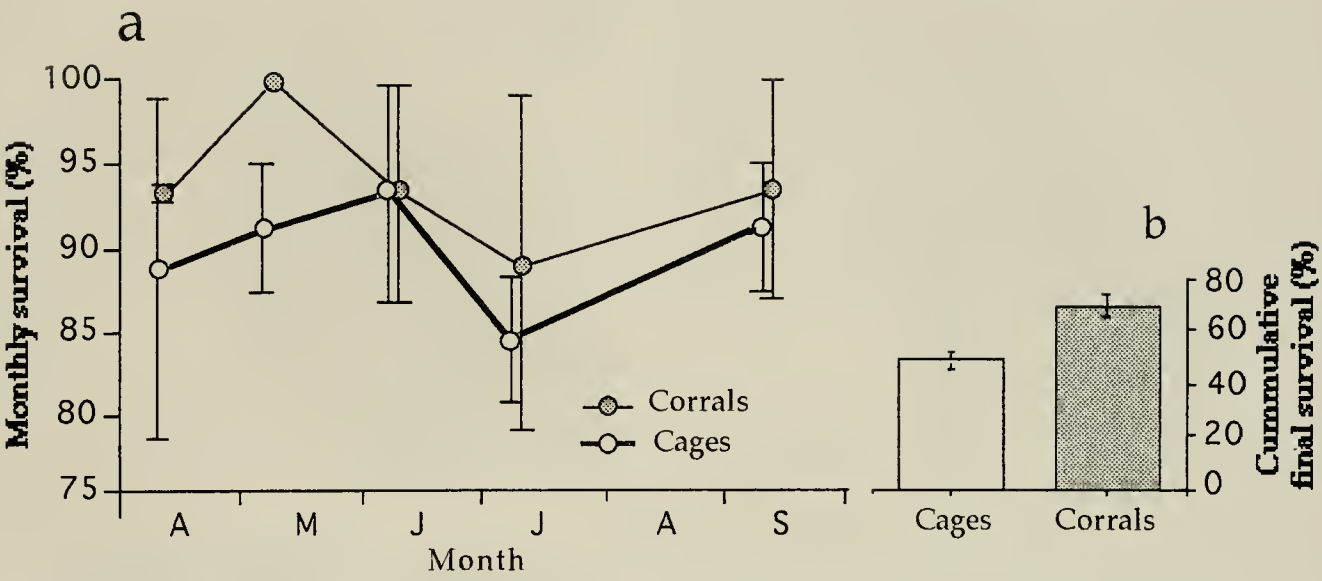

Figure 3. Monthly (a) and cumulative survival (b) of *Euvola ziczac* in bottom culture (vertical bars represent the standard deviations of the values).

for the scallop *Euvola ziczac* may be obtained. Thus, the size range in adults located on natural banks is between 65 and 95 cm (Himmelman and Lodeiros, unpublished data), and sizes for individuals reared both in corrals (73 mm) and in cages (70 mm) were within this size interval. These sizes of scallop had wet muscle weights of 7–8 g, which are considered excellent for scallop commercialization (Dore 1991).

The rate of growth observed in this study (approx. 0.16 mm d^{-1}) was similar to that observed in *Euvola ziczac* (approx. 0.15 mm d^{-1}) for Velez et al. (1995) in the same locality, for a similar period of year (70% of the same period) and in the same period of 190 days, but with a higher initial high density (64 individuals m^{-2}) than in this study (15 individuals m^{-2}). These similar growth rates, despite the different densities in both studies, suggest that the growth observed in this study was not more influenced by the density used. This also suggests that the bottom culture productions of this species can be increased with the use of higher densities in the methods studied.

The high escape rate of individuals with the barrier-free method led to the discontinuation of this method. This suggests that *Euvola ziczac* has a high dispersion capacity, which would lead to a low recovery rate of the stock originally used for cultivation with no barriers. Therefore, we considered that it is necessary to develop a new experiment with a more adequate scale before suggesting the use of this barrier-free method. In any case, in other countries, such as Canada, Japan, and France, the use of the barrier-free bottom culture method in more adequate scales had a low recovery rate of the initial stock because of the high escape rates of scallops (Wildish et al. 1988, Aoyama 1989, Cliche et al. 1994, Dao et al. 1995). Nevertheless, Wang et al. (1992) showed that even when the recovery rate of the initial bottom stock was on the order of 54%, the low production costs exerted an influence on the high profitability of scallop cultivation of *Chlamys farreri*. In our case, the recovery rate of individuals with the barrier-free method was 16%. This may be considered as very low if we take into account that it was obtained after only 30 study days. This escape capacity was also evident in the corrals where, despite 1-m high barriers, escape gradually increased until the end of the study (36% escape). Furthermore, it was observed that some specimens cultured with this method, when unintentionally disturbed for the purpose of taking samples, showed a clearly evident capacity to escape beyond the 1-m high barriers. In these observations, we noted the increase in the vertical displacement capacity of *Euvola ziczac* as size increases. However, we do not exclude the possibility of the increase in the rate of depredation by some fish, octopus, and crab decapods during the time of the experiment. Nevertheless, this phenomenon was not noted in the course of our frequent observations.

At the end of the study, the cage-reared individuals presented a significantly lower survival rate than those reared in corrals. In the cages, greater protection from predators was expected because of the presence of netting on all sides that, theoretically, would impede their entrance of the same. In the cages, however, several fragmented shells were collected, a fact that indicated the action of predators. For this reason, a detailed search was conducted, and the presence of decapod crab juveniles *Calappa cinerea* was discovered. These had gone unnoticed until that point because of their strategy of burying themselves in the substrata. This decapod has strong chela that allow it to fragment *Euvola ziczac* shell, judging by the condition of the shells. This ability has also been noted in the species *Calappa ocellata*, as a result of its preying action on the bivalve *Brachidontes domingensis* (Hughes and Elner 1989). This suggests that the decapod *C. cinerea* apparently entered the cages at its juvenile stage, when the opening in the mesh still made this possible, so that it was also able to take advantage of the protection afforded by the cage. This situation helped avoid competition for food and being preyed upon. One observation that supports this hypothesis is that in the corrals, where there was no upper netting, the dead individuals of *Euvola ziczac* showed no shell fragmentation and nor were any detected *C. cinerea*. These observations differ from those for the cultivation of temperate water scallop species where the use of nets substantially decreased predation (Morgan et al. 1980, Quayle and Newkirk, 1990).

Because this study was conducted in a certain season of the year, possible biocontrol of decapod *C. cinerea* juveniles may not be present throughout the year. Furthermore, one of the predators that may possibly exert a dramatic effect on scallop survival under

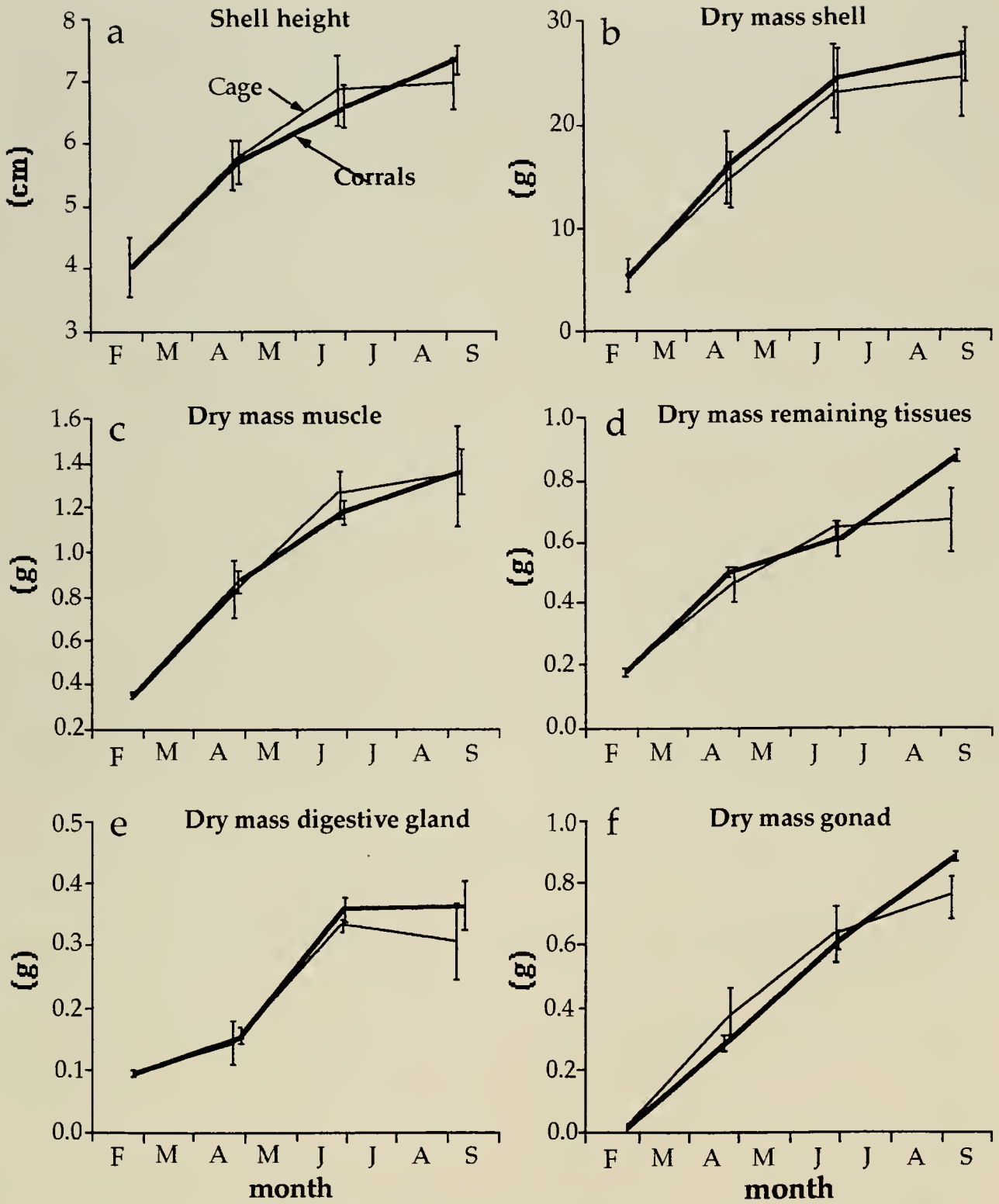

Figure 4. Growth in shell height (a) and dry mass of the shell (b) muscle (c), remaining soft tissues (d), digestive gland (e), and gonad (f) of the cultivated specimens of *Euvola ziczac* from bottom culture (vertical bars represent the standard deviations of the values).

bottom culture conditions, as noted on natural scallop banks, are the cephalopods *Octopus* spp. (Freites, personal observations). Nevertheless, despite the fact that the period of greatest influence by these predators fell within the experimental period of this study (from June to September), they were not observed. For this reason, in future studies aimed at determining the effect of predators on the survival of bivalves in corrals, it is advisable to cover different periods or seasons of the year.

Also, in the Golfo de Cariaco, hanging culture of these bivalves does not guarantee a lesser impact of predation compared to some

temperate water species (Quayle and Newkirk 1990, Hickman 1992). This is because of the recruitment of some predatory decapod and gastropod species during their planktonic larval stage, which allows them to gain access to the hanging baskets. Once inside these baskets, if uncontrolled, their growth is so fast that, in some cases, they have caused substantial mortality rates (>60%) in the cultivation of several bivalve species with culture potential, including *Euvola ziczac* (Freites et al. 1995, Freites et al. 2000), *Pinna carnea* (Narvaéz 1999) and pearl oyster *Pinctada imbricata* (Pico D., unpublished data).

The growth pattern for the corral and cage-reared individuals was similar, except in the latter sampling period, when the cage-reared individuals showed lower growth rates. These differences may not be attributable to a differential colonization by fouling organisms in nets of the enclosures or on shell that may, in the long term, affect food availability for the scallops, because the nets in both enclosures were cleaned throughout the experimental period because of the action of "grazing" of some fish and benthonic invertebrates on the net of the corrals and cage (personal observation), and because virtually no organisms colonized the shells of scallops in both enclosure, probably because the scallops were usually recessed in the sand. This together with maintaining the same density of individuals in the enclosures suggests that food availability was not a factor in the decreased growth observed in the cages.

One possible explanation is based on the fact that, as the *Euvola ziczac* individuals reared in cages increased in size and even when new *C. cinerea* decapod recruits were observed, they were physically unable to prey on the scallop because of the larger, more resilient shell, as evidenced by the subsequent lack of fractured shells. We do not rule out the fact, however, that the decapod juveniles may cause some disturbance leading to a defensive behavior, so that the bivalves close their valves, thus restricting filtration time and, consequently, affecting growth.

As we have seen earlier, the growth of juvenile scallops reared in the two types of enclosures may not be used as a selection criterion for recommending the use of dismissal of one of these two types of enclosures studied, particularly if we take into account that at the end of the experimental period, no significant differences were noted in muscle weight. Survival, however, may be used as a selection criteria, because, in the case of corrals, the rate was 27% higher. This difference would significantly affect the production level of the culture in favor of corrals. Furthermore, corrals involve a lower investment cost, and it is likely that operational costs would also be lower, because of a need for less material to construct the enclosure, and while seeding, supervision and harvesting tasks are easier. Taking the above into account, the use of corral-type enclosures is advisable, with a height of over 1 meter, to minimize escape.

Finally, during this study, an average growth rate of 6 mm month^{-1} was found. This is high if we compare it to growth rates of other scallop species of commercial importance, such as *Pecten maximus* (2 mm month^{-1}), *P. sulsicostatus* (2 mm month^{-1}), *P. albicans* (3 mm month^{-1}), and *P. novaezelandiae* (4 mm month^{-1}) (Mottet 1979). Only *Amusium balloti* (Williams and Dredge 1981), *Chlamys purpuratus* (DiSalvo et al. 1984), and *Argopecten circularis* (Felix-Pico 1991) scallops attained similar rates of growth. In this manner, the growth rate of bottom-reared *Euvola ziczac*, its survival and relatively low cost with this culture method (Ventilla 1982) offer clear possibilities for further investigations in the development of commercial culture of this species.

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ALLOZYME AND BIOCHEMICAL VARIATION AT THE OCTOPINE DEHYDROGENASE LOCUS IN THE SCALLOP *EUVOLA ZICZAC*

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ABSTRACT High activities of octopine dehydrogenase (Odh) in the adductor muscle of bivalve molluscs are associated with a dependence on anaerobic glycolysis during swimming. The Odh locus is polymorphic in the scallop *Euvola ziczac*. Estimated apparent Odh K_ms for arginine were not different among nine Odh genotypes: however, K_ms for pyruvate differed significantly ($P < 0.001$) between heterozygous and homozygous scallops. The estimated apparent K_m values of Odh for arginine and pyruvate are dependent on their respective cosubstrate concentrations. Possible mechanisms for this overdominance include null alleles, aneuploidy, and higher fitness of the heterozygous. Our data suggest that heterozygous superiority in fitness is the most likely explanation for the apparent overdominance at the *Odh* locus.

KEY WORDS: Arginine, bivalves, molluscs, octopine dehydrogenase, overdominance, pyruvate, scallops

INTRODUCTION

Numerous studies demonstrate how allozymes influence various components of fitness. For example, allozymes differ in their catalytic properties, including kinetic parameters (K_m and V_{max}), and heterozygous genotypes may show overdominance (exceeding the two corresponding homozygous genotypes) (Sarver et al. 1992); be intermediate in catalytic efficiency between homozygous genotypes (Hoffmann 1981, 1983); or exhibit dominance, having catalytic efficiencies similar to the most efficient genotype (Hillbish and Koehn 1985; Nirchio et al. 1991).

Allozyme heterozygosity and growth rate are positively correlated in many bivalve species (Beaumont and Zouros 1991; Zouros et al. 1992; Hedgecock et al. 1996). Higher levels of heterozygosity are associated with a lower basal metabolic rate that allows heterozygous individuals to devote more of their aerobic scope to growth and reproduction (after meeting basal requirements) (Koehn and Shumway, 1982). Recently, Hedgecock et al. (1996) found in the Pacific oyster *Crassostrea gigas* (Thunberg) that not only are oxygen consumption rates lower for hybrid compared with the inbred larvae, but also the net efficiency of protein synthesis is much higher for the hybrids. Several authors (Garton et al. 1984; Rodhouse et al. 1986; Volckaert and Zouros 1989) have suggested that organisms use this energy surplus for functions that increase fitness. In sedentary molluscs such as mussels and oysters, metabolic energy would be better invested in growth during the juvenile stages and in reproduction in adults. However, scallops are active bivalves that avoid predation by vigorous swimming; thus, selection of an allozyme could result in an increased locomotion capacity.

Scallops display sudden bursts of muscle activity, initially sustained by arginine phosphate breakdown, followed by the activation of glycolytic pathways that result in rising levels of octopine (Chih and Ellington 1983; Bricelj and Shumway 1991). Octopine is produced by the reductive condensation of arginine and pyruvate catalysed by octopine dehydrogenase (Odh, EC 1.5.1.11), in the presence of NADH. High activities of Odh occur in the adductor muscles of scallops (Chih and Ellington 1983; Alfonsi et al. 1995). The advantage of octopine formation in adductor muscles may be that oxidation of NADH removes arginine and thereby facilitates the formation of ATP from arginine phosphate.

The energy needs among molluscs vary, as scallops, which swim, require higher instantaneous rates of ATP production than sedentary bivalves such as mussels and oysters. Phosphoarginine is the principal fuel during valve snapping, and octopine accumulates during the subsequent recovery phase under functional anaerobiosis (Bricelj and Shumway 1991). Genetic effects on glycolitic ATP production are correlated with increased ability for burst activity in pectinids. Volckaert and Zouros (1989) found in the scallop *Placopecten magellanicus* (Gmelin) that heterozygosity and octopine accumulation after burst activity are correlated. The degree of heterozygosity and the maximal activity of pyruvate kinase and Odh are positively correlated in the adductor muscle of the scallop *Euvola ziczac* (Alfonsi et al. 1995).

In scallops, the primary function of Odh is to maintain the redox balance of the muscle during exhaustive exercise. The present study was designed to determine whether allozymes of Odh, which is polymorphic (Coronado et al. 1991), differ in catalytic properties. We determined this by measuring the apparent K_m of Odh in the scallop, *E. ziczac*, under varying concentrations of pyruvate and arginine.

MATERIALS AND METHODS

Adult scallops, *E. ziczac* ($n = 103$), were collected in 1998 during their sexual resting period from the waters of the Gulf of Cariaco (Chacopatica) on the northeastern coast of Venezuela (10°30′10″N, 64°13′06″W). They were maintained in running aerated seawater.

To determine the genotype at the Odh locus, the adductor muscle from each individual was excised, minced, and centrifuged, and the supernatant was analyzed by horizontal 12% starch gel electrophoresis. The activity of Odh was identified using the staining procedures described by Morizot and Schmidt (1990). Allelic variants were designated by letters, with "a" being always the most anodic. To prepare the enzyme extracts, the frozen adductor muscle of each specimen was chopped and homogenized in 20% w/v cold 50 mM imidazole-HCl buffer in ice, pH 7.5, with 2 mM ethylenediaminetetra-acetic acid. The homogenized tissue was

centrifuged at 27,000 *g* for 20 min at 4 °C. Solid ammonium sulphate was added to the supernatant to reach 70% saturation. The resulting suspension was stirred at 4 °C for 30 min and then centrifuged at 20,000 *g* for 20 min. The pellet was dissolved in a small volume of the homogenizing medium, applied to a Sephadex G-100 column equilibrated with 50 mM Tris-HCl (pH 7.6) at 24 °C, and eluted with the same buffer. The eluted fraction with highest Odh activity was used for kinetic analyses.

Odh activity was measured by recording changes in optical density (OD; 365 nm) that were caused by the oxidation of NADH. Reactions were run using 25 µL of the enzyme preparations in 1.25 mL of incubation mixture. The routine enzyme assay for maximal activity was 0.2 mM NADH, 2.5 mM pyruvate, and 5.0 mM arginine in a 50 mM imidazole buffer at pH 7.5. All of the assays were run at 24 °C. The enzyme activity was expressed as spectrophotometric units (OD). Maximal activity was recorded between pH 6.0 and 7.5 in pilot enzymatic assays.

Odh followed Michaelis-Menten kinetics for both arginine and pyruvate, at saturation concentrations of the other substrate and of NADH. Substrate inhibition by pyruvate was observed at concentrations over 2.5 mM. Accordingly, the apparent Michaelis constants (apparent K_m) for the substrates arginine and pyruvate were estimated from the Michaelis-Menten equation, according to Churchill and Livingstone (1989):

$$\text{Estimated apparent } K_m = (V_{max}/V) - 1/S$$

where *V* represents the initial reaction velocity at either a pyruvate or arginine subsaturating concentration, when the respective cosubstrate was at a saturating level. Before applying this formula, the maximal velocity was calculated from the Lineweaver-Burke plots (Segel 1975), in which the concentration of one substrate (A) was varied and the concentration of the other substrate (B) kept constant. The data were fitted to the following equation:

$$1/V = K_m^B/V_{max} (1 + K_m^A/A)\ 1/B + 1/V_{max}$$

The initial velocity was recorded against the pyruvate concentration (0.10, 0.20, 0.83, and 2.50 mM) at fixed arginine concentrations (0.5, 1.5, 3.0, and 5.0 mM). The inverse of the initial velocity (1/V) was plotted against the inverse of the pyruvate concentration (1/S) for each arginine concentration. The Y intercepts of the Lineweaver-Burke lines estimated by linear regression analysis were plotted against the inverse of arginine concentration. The maximal velocity was determined from the value of the resulting Y intercept, which was essentially similar to that estimated by the routine enzyme assay. Likewise, the data were plotted as a function of the arginine (0.5, 1.5, 3.0, and 5.0 mM) concentration. The V_{max} value estimated agrees with that obtained using varying concentrations of pyruvate at fixed concentrations of arginine. Substrate inhibition of V_{max} was observed for pyruvate concentrations over 2.5 mM at each arginine concentration.

Deviations from expected values of allele frequency for Hardy-Weinberg equilibrium were tested by using a Chi-square analysis. The deficiency or excess of heterozygotes (analyzed by the F statistic) and the effective number of alleles at this locus (N_e, the reciprocal of the sum of squares of the allele frequencies) were calculated by using the statistical program Genes in Populations, version 2 (Perkins and Paul 1995).

RESULTS AND DISCUSSION

The sample of 103 individuals from the population of Chacopatica contained nine Odh genotypes: c/c, d/d, e/e, b/c, c/d, c/e, c/f, d/e, and d/f, determined by five alleles Odh^b, Odh^c, Odh^d, Odh^e, and Odh^f. Because genotypes that include alleles a and b are very rare (Table 1), it was not possible to obtain sufficient samples to study their catalytic properties of these rare alleles. Allele frequencies have been stable since the first sample was examined in 1984. All three samples were in Hardy-Weinberg equilibrium. Heterozygote superiority probably provides the best explanation for the maintenance of the polymorphism.

Apparent K_m for arginine and pyruvate were related to variations in the concentrations of the respective cosubstrates, because K_m decreased as the concentration of cosubstrate increased (Table 2). This suggests a mechanism that favors the formation of octopine when the concentration of the two substrates increases simultaneously, as is seen in active individuals. The availability of arginine and pyruvate could be the two limiting factors in the regulation of Odh activity for maximal glycolytic capacity during the escape response and recuperation of *E. ziczac*. In addition, specific genetic influences affect the regulatory properties of the enzyme by acting on their relative substrate affinities.

Results for the K_m of pyruvate and arginine at different cosubstrate concentrations were separated into two groups: homozygotes and heterozygotes. Table 3 indicates no significant differences for the K_m of arginine (pyruvate as cosubstrate) ($F = 0.017$; $P > 0.05$), whereas highly significant differences were detected between homozygotes and heterozygotes for the K_m of pyruvate (arginine as cosubstrate) ($F = 29.33$; $P < 0.001$). These results indicate that the affinity of the Odh enzyme for pyruvate was predominantly greater in heterozygous than in homozygous individuals. Similar results were observed by Walsh (1981) for three phenotypes of Odh in the anemone *Metridium senile* (L.), in which the heterozygotes showed a higher affinity for pyruvate. Sarver et al. (1992) mea-

TABLE 1.

Allele frequencies, effective number of alleles (N_e), observed (H_o) and expected (H_e) values for heterozygosity, and values of γ^2 are tests of goodness of fit to Hardy-Weinberg proportions for the Odh locus in samples of Chacopatica collected in 1984, 1994, and 1998.

	Allele Frequency												
	a	b	c	d	e	f	*N*	N_e	H_o	H_e	F_{is}	γ^2	*P*
1984*	0.011	0.033	0.456	0.244	0.244	0.011	45	3.05	0.533	0.609	0.12	8.82	>0.1
1994†	0.000	0.000	0.500	0.310	0.150	0.050	113	2.75	0.655	0.636	0.03	4.76	>0.1
1998	0.000	0.005	0.495	0.311	0.165	0.024	103	2.66	0.621	0.631	0.014	3.72	>0.3

N = sample size. F_{is} indicates deficiency or excess of heterozygotes.

* Coronado et al. 1991.

† Fernandez 1995.

TABLE 2.

Means and standard deviations for the estimated apparent K_m for both substrate arginine and pyruvate for the different genotypes, at the different concentrations.

		Cosubstrate Arginine (mM)			Cosubstrate Pyruvate (mM)		
Genotypes	*N*	1.5	3.0	5.0	0.025	0.83	2.5
bc	1	0.25	0.18	0.11	0.91	0.83	0.42
cc	7	1.70 ± 1.15	0.58 ± 0.14	0.35 ± 0.09	2.80 ± 1.38	1.61 ± 0.60	0.82 ± 0.29
cd	7	1.20 ± 0.33	0.57 ± 0.17	0.19 ± 0.07	1.50 ± 0.59	1.18 ± 0.47	0.61 ± 0.21
ce	7	1.52 ± 0.62	0.48 ± 0.15	0.25 ± 0.08	4.60 ± 1.60	1.99 ± 0.61	0.99 ± 0.35
cf	1	0.19	0.13	0.06	2.39	1.44	0.76
dd	7	2.33 ± 1.44	1.04 ± 0.27	0.42 ± 0.11	2.48 ± 1.65	1.71 ± 0.93	0.83 ± 0.33
de	7	0.67 ± 0.34	0.35 ± 0.09	0.17 ± 0.04	2.41 ± 1.15	1.31 ± 0.46	0.67 ± 0.19
df	4	0.49 ± 0.21	0.26 ± 0.11	0.16 ± 0.06	1.86 ± 0.63	1.40 ± 0.43	0.59 ± 0.16
ee	3	0.69 ± 0.33	0.22 ± 0.05	0.14 ± 0.02	3.56 ± 1.24	1.71 ± 0.52	0.53 ± 0.11

N is the number of animals examined.

sured specific Odh activities in a large number of individuals of the mussel *Mytilus trossulus* Gould for Odh and found that the mean Odh activity was greater in heterozygotes than homozygotes.

Multiple range analysis (least-significant difference) indicate three groups in increasing order of K_m: (1) d/f, e/e, d/e, c/d, and c/e; (2) c/d, c/e, and c/c; and (3) d/d. Genotypes c/d and c/e belonged to the groups with higher and medium affinities [1] and [2], Table 3). By increasing Odh affinity for pyruvate, heterozygous individuals could enhance the ability of the muscle to maintain the NADH/NAD^+ redox balance during the glycolytic flux which can occur during high-intensity muscle work. This affinity would be particularly useful during the initial phase of glycolysis when pyruvate concentration is low and arginine levels begin rising (arginine phosphate pool is depleted). Moreover, the shunting of pyruvate to mitochondrial metabolism or cytoplasmic synthesis of alanine could be inhibited to rapidly meet the energy demands under functional anaerobiosis of the contracting fibers. On the other hand, it appears that arginine does not represent a control or limiting factor for the anaerobic glycolitic capacity for the fast muscle contraction of *E. ziczac*. This assertion, however, does not exclude the possibility of other genetic influences on the enzymatic conversion of arginine into arginine phosphate, which would assure a faster recuperation after a burst exercise.

Heterozygotes show an apparent overdominance in K_m for pyruvate that may be a fitness component if the concentration of pyruvate is low. Apparent overdominance (heterozygous genotypes are phenotypically superior to homozygous genotypes), in fitness components such as growth, viability, and fecundity, has been observed in many species of marine bivalves (Sarver et al. 1992). Possible explanations for overdominance, as well as the commonly reported deficiencies of heterozygotes, include null alleles (Foltz 1986) and aneuploidy (Thiriot-Quievreux et al. 1988). Several studies of allozyme inheritance have found substantially higher frequencies of null alleles in bivalves than found in other organisms, suggesting that null alleles or segmental aneuploidy may play a role in the apparently lower fitness of allozyme heterozygotes (Gaffney 1994). Null alleles or missing chromosomes (and therefore missing alleles) could contribute to fitness advantages of heterozygotes. In *Mytilus edulis* (L.), Hoare and Beaumont (1995) found not only heterozygotes, but also homozygotes for a null Odh allele. We believe this situation unlikely to occur an active species, such as *E. ziczac*.

TABLE 3.

Means and standard deviations from the estimated apparent K_m for pyruvate for heterozygous and homozygous individuals.

		Cosubstrate Arginine (mM)		
Genotype	*N*	1.5	3.0	5.0
Heterozygous	27	0.72 ± 0.49	0.32 ± 0.16	0.16 ± 0.06
Homozygous	17	1.57 ± 0.68	0.61 ± 0.33	0.30 ± 0.12
		Cosubstrate Pyruvate (mM)		
		0.205	0.83	2.5
Heterozygous	27	2.84 ± 1.30	1.49 ± 0.36	0.75 ± 0.16
Homozygous	17	2.64 ± 1.16	1.66 ± 0.33	0.83 ± 0.2

N is the number of animals examined. Means are different ($P < 0.001$).

Considering an alternate explanation of heterozygote superiority in fitness or other phenotypic attributes to explain our results, there are two possible scenarios, as follows.

(1) Individuals that appear single banded (homozygous), which were numerous in our sample, may really be heterozygous (active/null alleles); if so, we would expect a bimodal distribution, with a resulting higher variance of activity in homozygous compared with heterozygous. However, this was not the case for our samples of *E. ziczac* (see Table 3).

(2) Results obtained in the analysis of other enzymes (pyruvate kinase, glucose 6-phosphate dehydrogenase, isocitrate dehydrogenase, and malate dehydrogenase) in *E. ziczac* indicate that their specific activity is correlated positively with heterozygosity. Because the Odh activity in homozygotes and heterozygotes scallops increases with heterozygosity at multiple loci, it seems highly unlikely that null alleles (or missing chromosomes) would occur at all of these loci (Alfonsi et al. 1995).

Therefore, our results can be best explained by assuming an overdominance at the Odh locus, which could enable the heterozygotes to increase their ability to escape from predators.

In conclusion, the pyruvate affinity of the adductor muscle Odh allozyme in *E. ziczac* appears to be a catalytic target upon which genetic influences act to determine the tissue's capability for maintaining a steady NADH/NAD ratio, that would support the rate of anaerobic glycolysis at the burst working muscle, such as the

sudden escape behavior commonly observed among scallops when they flee from predators. During routine work, we have observed that *E. ziczac* scallops are easily induced to vigorously snap their valves (swimming) when approached by gastropods, crabs, starfish, and human divers. This predator-avoidance behavior may be repeated for several minutes before entering in a variable resting period. However, the nature of the relationship that exists between the capability to sustain muscle contraction in response to predatory stimulus and the Odh genotypic variants in *E. ziczac* is not clear.

Finally, further research is required on some biochemical and physiological events associated with activity in different genotypic variants of *E. ziczac*, both under laboratory bioassays and field work conditions, because predation is a significant component in the life history of adult scallops, we are currently searching for a possible relationship between the escape reaction and the different genotypes, and because polymorphism at the Odh locus in *E. ziczac* seems to be important for understanding genetic variation in molluscs, we are also searching for the presence of polymorphism in other, sedentary and motile, species of molluscs. Additionally, we are examining strombine and alanopine dehydrogenase (which also serve as hydrogen and carbon sinks in maintaining redox balance) in several species of molluscs during anaerobic metabolism, for possible polymorphisms and their maintenance mechanisms.

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ENVIRONMENT AND POPULATION ORIGIN EFFECTS ON FIRST SEXUAL MATURITY OF CATARINA SCALLOP, *ARGOPECTEN VENTRICOSUS* (SOWERBY II, 1842).

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ABSTRACT Two populations of catarina scallop, and their cross (F1), were evaluated for the age at first sexual maturity, and for their gonadal development in both populations' native environments. All experimental groups were hatchery produced. Differences in mean gonad index (MGI) were seen between the different environments. In Bahia Magdalena, a site characterized by high productivity and lower average water temperatures, the MGI was higher than for scallops grown in Bahia Concepción, a bay with lower productivity and higher average water temperatures. Differences in age and size at first sexual maturity, defined as those when 50% of the scallops in any group were sexually mature, were seen between the populations when grown at Bahia Magdalena but not when grown at Bahia Concepción. At Bahia Concepción, none of the groups had reached their first sexual maturation after the experimental period of 7 mo. At Bahia Magdalena, the Magdalena population and the F1 reached sexual maturity at an early age of 4 mo, whereas the Concepción population reached maturity when 5 mo old. Also for the Bahia Magdalena environment, the gonad index (GI) estimated at first sexual maturity for the Magdalena population and the F1 was significantly higher than that estimated for the Concepción population at that same age. There were no significant differences in GI values when the groups were grown at Bahia Concepción. The differences between populations in age at first sexual maturity suggest that a triggering mechanism exists in catarina scallop for the initiation of sexual maturation, whereas the differences between environments suggest that regardless of that mechanism, environmental conditions have a significant role in further maturation processes.

KEY WORDS: *Argopecten ventricosus*, environment, gonad index, populations, maturation

INTRODUCTION

The catarina scallop, *Argopecten ventricosus* (Sowerby II, 1842), which is a functional hermaphrodite species, is an important fishery and aquaculture resource on both coasts of the Baja California Peninsula, Mexico. The geography of the peninsula results in this species distributing and growing in different environmental conditions: semitropical to temperate on the Pacific Ocean side and tropical on the Gulf of California side. Because of this, as well as the presumed isolation caused by the Peninsula barrier itself, natural populations existing on both sides are expected to be genetically different, that is, to have evolved differently in response to environmental conditions on each side. In fact, we have demonstrated that there are differences between these two populations in growth and survival (Cruz and Ibarra 1997, Cruz et al. 1998).

An additional important trait to compare in populations on both sides of the peninsula is the age and size at which each population reaches its first sexual maturity. It has been stated that the reproductive cycle of scallops is a genetically controlled response to environmental conditions (Sastry 1970, Sastry 1979, cited by Barber and Blake 1991), which depends on the optimum interactions between exogenous and endogenous factors. When the appropriate combination of exogenous and endogenous factors occurs, a minimum age (or size) has to be reached before the beginning of gametogenesis (Barber and Blake 1991). Differences in the onset of sexual maturity and reproductive cycle have already been reported for other bivalve species (Dalton and Menzel 1983, Knaub and Eversole 1988, Barber et al. 1991, Mackie and Ansell 1993). Among different bivalves studied simultaneously at different sites, or through transplantation, there are differences in the onset of gametogenesis (Newell et al. 1982, Barber and Blake 1983, Walker and Heffernan 1994), spawning time (Brousseau 1987, Emmett et al. 1987, Paulet et al. 1988), fecundity (Bricelj et al. 1987), and gametogenic cycle (Wilson 1987, Thorarinsdóttir 1993, Sbrenna and Campioni 1994). Some of the previously reported differences are not necessarily caused by genetic factors but by different environmental conditions at each site studied.

Different studies with catarina scallop have been performed regarding sexual maturation and gametogenic cycles (Baqueiro et al. 1981, Tripp-Quezada 1985, Villalejo-Fuerte 1992, Félix-Pico 1993, Villalejo-Fuerte and Ochoa-Báez 1993). However, differences between populations in age at first sexual maturity or in the effects of different environments on gametogenic cycles and maturation have not been investigated.

In this study, we evaluated the onset of first sexual maturity and the gametogenic cycles for two populations of catarina scallop and their cross (F1). The two populations were Concepción in Bahia Concepción on the Gulf of California side of the Baja California, and Magdalena in Bahia Magdalena on the Pacific Ocean side of the Baja California peninsula. All groups, Magdalena, Concepción, and their F1s, were simultaneously evaluated in both environments.

MATERIALS AND METHODS

Populations and F1

Spawners used, conformation of the experimental groups, and larvae rearing have been described by Cruz and Ibarra (1997). In summary, four groups were produced by mass spawning; Magdalena, Concepción, and both reciprocal F1s. At a spat size of 1.5 cm length and 2.5 mo old, 9 (pseudo) replicates, each with 100 scallop spats, were formed within each group by randomly sampling 900 spats from the total group pool.

Grow-Out

The spat contained within each of the nine replicates per group were simultaneously transported to each of the experimental field areas, Bahia Magdalena and Bahia Concepción, where they were maintained for 5 mo. At each site, the scallops contained in each replicate were kept in a Nestier tray suspended from a long-line for 45 days and then were transferred to bottom culture to avoid position effects on growth caused by water temperature stratification. Nestier trays were attached to a metal structure anchored to the bottom. Maintenance was performed monthly. Densities were the same for all replicates within groups and in both environments (Cruz et al. 1998).

Gonad Sampling and Histology Analysis

Sampling for gonad tissue began after 1.5 mo of grow-out, at 4 mo age. Three individuals were sampled per replicate (27 per group) at ages 4, 5, 6, and 7 mo. Samples were fixed in Davidson's fixative and were preserved in 70% alcohol. The hematoxylin-eosin staining technique was used. Sexual maturity was evaluated with a modified Villalejo-Fuerte (1992) scale for this hermaphrodite scallop, where seven stages are included for the female portion of the gonad (Stage 0 = undifferentiated or virginal; Stage I = resting; Stage II = start of gametogenesis; Stage III = advanced gametogenesis; Stage IV = maturity; Stage V = spawned; Stage VI = postspawned).

Age-Size at First Sexual Maturity

Age and size at first maturity were established by a different criterion than that commonly used when populations are evaluated following a field-born cohort (Nikolsky 1969). Under that methodology, age-size at first maturity is estimated when the cumulative frequency of mature individuals reaches 50% in the cohort. Field-born individuals of a population are of different ages because spawning of the whole population usually last from days to weeks. In the present study, all individuals were the same age. Therefore, in this study, age and size at first sexual maturity were defined as the age when 50% of the organisms within any group were in the "maturity" gonadal stage, or Stage IV as defined above, or when the sum of individuals in Stage IV (maturity), Stage V (spawn), and Stage VI (post-spawn) was ≥ 50%. Only the female gonad portion was used for the establishment of the age and size at sexual maturity.

Gonad Indices

Gonad indices (GIs) were calculated for each replicate within each group based on a calculation by Seed (1976) by using the number of individuals and the stage at each age (4, 5, 6, and 7 mo) to find a GI at age for each group as follows:

$$GI_{ijk} = [(0*N_0) + (1*N_I) + (2*N_{II}) + (3*N_{III}) + (4*N_{IV}) + (5*N_V) + (6*N_{VI})]/N_{tot(ijk)}$$

where GI_{ijk} is the GI for replicate i (i = 1,2,3,...9), of the group j (j = 1,2,3), in the environment k (k = 1,2); $N_{subscript}$ is the number of individuals in that gonadal stage for replicate i; and $N_{tot(ijk)}$ is the total number of individuals in that replicate of that group in that environment.

Statistical Analyses

The GIs estimated for each replicate within the groups were analyzed by a complete two-factor, Model I, analysis of variance, where age was taken into consideration as a covariable. After establishing the lack of differences between the reciprocal F1s ($P > 0.05$), for all further analysis the F1s were pooled into what is defined as the F1 between these two populations. The effects of group (Magdalena, Concepción, and F1), environment (Bahia Magdalena and Bahia Concepción), and their interaction on GI were analyzed. Effects means were compared with a Tukey test, setting $\alpha = 0.05$. Additionally, at the age when first sexual maturation was observed, as defined above, a second partial Model I analysis of variance was made. This was performed to establish the effect of groups and environments on GIs at the age of first sexual maturity and to find out whether there was a group by environment interaction for GI. All statistical analyses were performed using a computer software (Statistica, version 5; StatSoft, Inc.; Tulsa, OK), and significance for all analyses was set to $P < 0.05$.

RESULTS

First Sexual Maturity

At Bahia Magdalena, the age at first sexual maturity (Stage IV) for the Magdalena population was 4 mo. However, at this age, which corresponds to the first sampling time during grow-out (1.5 mo of grow-out), 56% of the individuals were scored as matured, but 9% were spawned, and 13% postspawned. This indicated that first sexual maturity occurred slightly before this time. Shell height at 4 mo of age was 20.0 mm (SD ± 0.88 mm). At this same age, the F1 also reached sexual maturity, as defined in this study, since it had 41% individuals in the maturity stage, 19% spawned, and 5% postspawned (Table 1). Shell height was 21.2 mm (SD ± 0.85 mm). At the age of 4 mo, the Concepción population had no mature or spawned individuals, but 4% were postspawned (Table 1). At 5 mo of age, the Concepción population had 75% mature individuals, reaching sexual maturity (Fig. 1) at a shell height of 32.9 mm (SD ± 1.34 mm).

At Bahia Concepción, sexual maturity was not reached by any group during the experimental period (Fig. 1). Although sexual maturity was not detected in this environment, a differential pattern between groups was evident from 5 mo to the end of the study: a larger percentage of individuals from the Magdalena population and the F1 were postspawned than the percentages seen for the Concepción population. Also, at 7 mo of age, corresponding to the last sampling date, 16% of the individuals within the Magdalena group and 4% of the F1 were already matured, whereas within the Concepción group there were no mature, spawned, or postspawned individuals (Fig. 1).

GIs

Both main effects (group and environment) were significant for both analyses, the whole grow-out period and the age (4 mo) at sexual maturity in the Magdalena population and the F1. There was no interaction between groups and environments (Table 2). For the whole grow-out period, mean GIs (MGIs) for all groups at Bahia Concepción (MGI 1.63) were significantly less than those at Bahia Magdalena (MGI 3.72) (Table 3). These MGI values indi-

TABLE 1.

Frequencies (in percentages), of *A. ventricosus* at 4 mo of age, in each gametogenic stage within each experimental group when grown at Bahia Magdalena and Bahia Concepción.

	Bahia Magdalena			Bahia Concepcíon		
Stage	Magdalena Population	F1	Concepción Population	Magdalena Population	F1	Concepción Population
0 Undifferentiated	0	5	22	75	89	100
I Resting	0	5	0	25	6	0
II Initial gametogenesis	0	5	48	0	5	0
III Advanced gametogenesis	22	17	26	0	0	0
IV Maturity	56[a]	41[a]	0	0	0	0
V Spawned	9	19	0	0	0	0
VI Postspawned	13	5	4	0	0	0

[a] Indicates whether sexual maturity of female gonad portion was reached for that group at this age.

cated that, over the grow-out period, scallops at Bahia Magdalena were between the advanced gametogenesis (Stage III) and spawned (Stage V) stages, whereas those at Bahia Concepción were between resting (Stage I) and initial (Stage II) gametogenic stages. Within environments and for the whole grow-out period, there were significant differences between groups only when grown at Bahia Magdalena, where the two populations were different, and the F1 was in an intermediate maturity stage, which is not different from either population. The largest GI was that of the Magdalena population (GI 4.02), followed by the F1 (GI 3.81) and the Concepción population (GI 3.33) (Table 3).

At 4 mo of age, when sexual maturity had occurred, the MGI at Bahia Concepción was lower (MGI 0.5) than that at Bahia Magdalena (MGI 3.17). There were significant differences between groups only in Bahia Magdalena, with no significant differences in GI between the Magdalena population (GI 4.08) and the F1 (GI 3.57), whereas the Concepción population had the lowest GI (GI 1.85) (Table 3). At Bahia Concepción, GIs were not

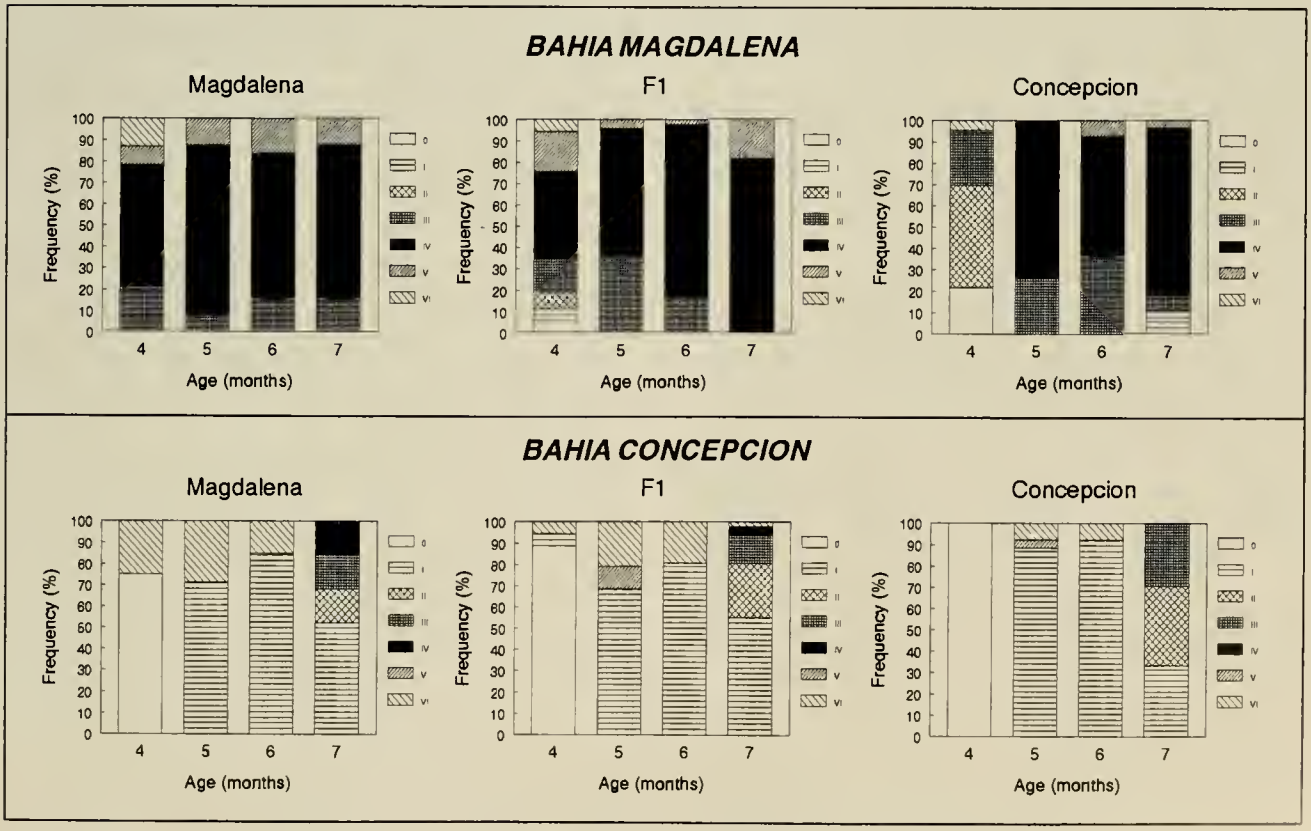

Figure. 1. Frequencies of gonadal developmental stages in *A. ventricosus* at the ages of 4, 5, 6, and 7 mo, for each experimental group at each environment. Stage 0 = undifferentiated; Stage I = resting; Stage II = start of gametogenesis; Stage III = advanced gametogenesis; Stage IV = maturity; Stage V = spawned; and Stage VI = postspawned.

TABLE 2.

Results of the analyses of variance testing significant effect on female GIs of *A. ventricosus* for the complete model during the grow-out period, and the partial model only at 4 mo of age (see Materials and Methods section).

Source of Variation	Full Model	Partial Model
Environment	0.0000[a]	0.0000[a]
Group	0.0015[a]	0.0069[a]
Interaction	0.8373	0.3340

[a] Indicates significance at the pre-established level of $P < 0.05$.

different between groups (1.0, 0.5, and 0.0, respectively, for the Magdalena population, F1, and Concepción population).

DISCUSSION

Differences between the two populations in age and size at sexual maturity were clearly evident when grown at Bahia Magdalena but not at Bahia Concepción. At Bahia Concepción, sexual maturity was not reached by any of the groups. However, at 7 mo, some mature individuals were already present for the Magdalena population, but not for the Concepción population. Previous work by Villalejo-Fuerte and Ochoa-Báez (1993) indicates that the native population at Bahia Concepción reaches sexual maturity at the age of 1 y and a 58-mm shell height. For other *Argopecten* species, as for example *Argopecten irradians*, the maximum gonad weight was reported to be at 57 mm shell height (Bricelj et al. 1987), whereas for *Argopecten gibbus*, ripe individuals as small as 20 mm shell height have been reported (Miller et al. 1979). In fact, precocious individuals such as those seen in the population of *A. ventricosus* from Bahia Magdalena have only been reported for *A. gibbus*, which reaches sexual maturation when only 71 days old (see review by Barber and Blake 1991).

The failure of all groups to reach sexual maturity during our experimental period when grown at Bahia Concepción can be explained by the environmental conditions which characterize this bay: low productivity (chlorophyll-*a* 0.38–1.63 mg/m^3) and high average annual temperature (24.9 °C) with a wider range (17.7–32.1 °C) (Martínez-López and Gárate-Lizárraga 1994, Reyes-Salinas 1994). Bahia Magdalena is characterized as a more benign environment. Average temperature is 22 °C, with a small range (20–26.6 °C) (Hernández-Rivas et al. 1993), and a high chlorophyll-*a* concentration (1.5–5.1 mg/m^3) (Acosta-Ruiz and Lara-Lara 1978). Poor environmental conditions are known to affect gonad development (i.e., decreases in reproductive output seen in *Placopecten magellanicus*) (Macdonald and Thompson 1985). Barber and Blake (1991) proposed that the oocyte reabsorption seen in different species of Pectinids could be caused by unfavorable temperatures that inhibit full gonad development. This was probably the case in the Bahia Concepción population, where despite the fact that mature individuals 4–6 mo old were not detected, there were some classified as postspawned. Furthermore, rather than in the undifferentiated stage, most scallops in Bahia Concepción were in a resting stage during most of the experimental period, which could have been caused by attempted maturation followed by follicular atresia because of high temperatures and low productivity.

TABLE 3.

GIs (SD) in *A. ventricosus*, for the whole grow-out period and for the age at sexual maturity (reached when grown at Bahia Magdalena at 4 mo of age) for each environment and for each experimental group.[a]

Environment	Group	Whole grow-out period	Age 4 mo (Sexual Maturity)
Bahia Magdalena	Magdalena GI	4.02 (0.45)^{a}	4.08 (0.75)^{a}
	F1 GI	3.81 (0.62)^{ab}	3.57 (0.94)^{a}
	Concepción GI	3.33 (1.14)^{b}	1.85 (1.28)^{b}
	BM MGI	3.72 (0.81)^{A}	3.17 (1.32)^{A}
Bahia Concepción	Magdalena GI	1.81 (1.26)^{c}	1.0 (2.24)^{c}
	F1 GI	1.75 (1.30)^{c}	0.5 (1.58)^{c}
	Concepción GI	1.34 (0.96)^{c}	0.0 (0.00)^{c}
	BC MGI	1.63 (1.22)^{B}	0.5 (1.54)^{B}

MGI (SD) is the average female GI of all groups within that environment. GIs for gonad by group are given. Means with the same letter within gonad part (female or male) are not significantly different. Group means differences within environment and sex in lower case. Capital case letters for differences between environments.

The mechanism that detains the maturation process under inadequate environmental conditions is not known. However, it is known that in *A. irradians*, the regulation of the gametogenic cycle is controlled by a neurosecretory cycle with a checkpoint that seems to act as a switching mechanism, allowing or delaying oocyte growth depending on food and temperature (Barber and Blake 1991). When scallops are subjected to prolonged threshold temperatures after the neurosecretory cycle enters the neurosecretory stage (NS) corresponding to cytoplasmic growth phase (NS III) or vitellogenesis (NS IV), scallops do not regress in NS, and vacuolization of cytoplasm and lysis of oocytes can occur (Sastry, 1966a, 1968, cited by Barber and Blake 1991). Whether a similar mechanism exists in the catarina scallop is not known, but it could explain the presence of atresias in scallops when they are grown in Bahia Concepción.

The differences between populations in age at sexual maturity suggest that a genetic triggering mechanism might exist for the onset of sexual maturity in the catarina scallop. When grown in Bahia Magdalena, an environment characterized by high productivity and lower temperatures, the mechanism of early maturation in the Magdalena population and the F1 is triggered, and because the prevailing environmental conditions at this site (low temperature and abundant food), full development is reached at an early age. A suggestion that the mechanism is genetically controlled comes from the age and size at which the two populations reached their first sexual maturity. The Concepción population reached sexual maturity in this environment at least 1 mo later than the Magdalena population. Inheritance (from the Magdalena population to the F1) in the dominant fashion of an early triggering mechanism is suggested by the F1 reaching sexual maturity at the same age as the Magdalena population and by the fact that the GIs of the F1 showed no significant differences with the Magdalena population at first sexual maturity. However, the GI of the F1 group was intermediate to the GIs of the two populations for the whole grow-out period, indicating more of an additive form of inheritance for this trait. Furthermore, whereas at Bahia Concepción sexual maturity was not reached for any group in this study, at 7 mo of age there were 16% mature individuals within the

Magdalena population, but only 4% mature and 2% post spawn for the F1 (6%). Further research, with segregation studies included, is required to provide a definitive answer to the inheritance of this trait. Whereas the inheritance of reproductive traits has been suggested in other mollusk species, no study has attempted to demonstrate it at the genetic level. For example, Knaub and Eversole (1988) found that the F1 between two populations of *Mercenaria mercenaria* resembled the paternal population in some reproductive traits and the maternal population in others. Also, each of two lines of *Crassostrea virginica*, derived from two populations 5–6 generations before, still followed the reproductive pattern of their original populations (Barber and Blake 1991).

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REPRODUCTIVE CYCLE OF THE RUGOSE PEN SHELL, *PINNA RUGOSA* SOWERBY, 1835 (MOLLUSCA: BIVALVIA) FROM BAHÍA CONCEPCIÓN, GULF OF CALIFORNIA AND ITS RELATION TO TEMPERATURE AND PHOTOPERIOD

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ABSTRACT This study describes, through monthly histological examinations of gonadal tissue samples, the reproductive cycle of *Pinna rugosa* and relates gametogenesis to temperature and photoperiod. Monthly gonadal samples were obtained from February 1993 to February 1994, in Bahía Concepción, Gulf of California, Mexico. Five stages of gonadal development were characterized: indifferent, developing, ripe, partially spawned, and spent. Histological evidence revealed hermaphroditism in 20.9% of animals sampled. Gametogenesis commenced in March, with ripe and spawning stages occurring from April to November, and no gametogenic activity occurring from December to February. From March to November, water temperature ranged from 20 °C to 31 °C, with an average range of light of 650–820 min/day. *P. rugosa* had a seasonal gametogenic cycle directly related to water temperature and photoperiod.

KEY WORDS: Reproduction, bivalve, *Pinna*, histology, Gulf of California

INTRODUCTION

The rugose pen shell, *Pinna rugosa* Sowerby, 1835, is commonly known in Mexico as "hacha" (hatchet). This bivalve is of commercial importance and supports a fishery in the northwestern area Gulf of California, Mexico. *P. rugosa* is greatly appreciated by consumers because of its tasty, large adductor muscle, commonly referred to as "callo." The pen shell fishery has been an important economic activity in Mexico for many years. Production trends, however, have drastically declined over the past years, and some populations have been depleted (Reynoso-Granados et al. 1996). Few biological studies of *P. rugosa* have been conducted (Arizpe and Félix 1986, Arizpe and Covarrubias 1987, Mazón-Suástegui and Avilés-Quevedo 1988, Ruíz-Verdugo and Cáceres-Martínez 1990, Arizpe 1995).

Documentation of the reproductive biology of *P. rugosa* in the Gulf of California is extremely scarce. Noguera and Gómez-Aguirre (1972) described the reproductive cycle of *P. rugosa* in Laz Paz Bay, B.C.S., Mexico, and they showed that gametogenesis commenced in mid-spring and that the animals spawned in late summer.

Because of the economic importance and high price obtained by the callo, efforts have recently been under way to cultivate this species. Therefore, studies of its reproductive biology are essential to achieve reproduction in a laboratory setting. This study documents the reproductive cycle of *P. rugosa* from Bahía Concepción, Gulf of California, Mexico, and examines the relationship of gametogenesis to temperature/photoperiod.

MATERIALS AND METHODS

Bahía Concepción, Mexico, is located on the western coast of the Península of Baja California, between 26°55′ and 26°30′N and 112° and 111°40′E. The bay is approximately 40 km long and 10 km in its widest part and oriented in a NW-SE direction (McFall 1968).

Monthly, between 13 and 35 specimens of rugose pen shell were collected by a scuba diver at a 2- to 8-m depth from February 1993 to February 1994. Animals were collected from a wild population located off Santispac Beach in Bahía Concepción, Gulf of California. The individuals were collected and fixed in 10% formalin solution. When the biological samples were collected, water temperature at the collection site was recorded.

The visceral mass (gonad included) was dissected from each pen shell and stored in 70% alcohol. Later, a slice of tissue of the dorsal area of the visceral mass was cut. This tissue samples were dehydrated in an ethanol series of progressive concentrations, cleared in toluene, and embedded in paraffin. Serial sections 7–9 μm thick were obtained with a rotary microtome. Preparations were stained with hematoxilyn and eosin. The gonad structure was examined under a microscope, and the sex was determined for each animal by the presence of egg or sperm in the tissue section.

Each tissue section of *P. rugosa* was categorized on the basis of the qualitative characteristics of five stages of maturation (indifferent, developing, ripe, partially spawned, and spent) as described by Villalejo-Fuerte and García-Domínguez (1998). The monthly relative frequencies of the stages of gonadal development throughout the annual cycle were obtained. This enabled the description of the reproductive cycle. The spawning season is defined as the time period containing ripe and partially spawned individuals.

To obtain a quantitative value that represents the reproductive activity, a monthly gonad index (GI) was computed (Heffernan et al. 1989) utilizing a numerical grading system. Three categories were established according to the degree of development of the gonad, with 1 = indifferent and spent, 2 = developing, and 3 = ripe and partially spawned. The monthly GI was determined by multiplying the number of specimens ascribed to each category by the category score, summing all such values, and dividing the resulting value by the total number of pen shells analyzed. The

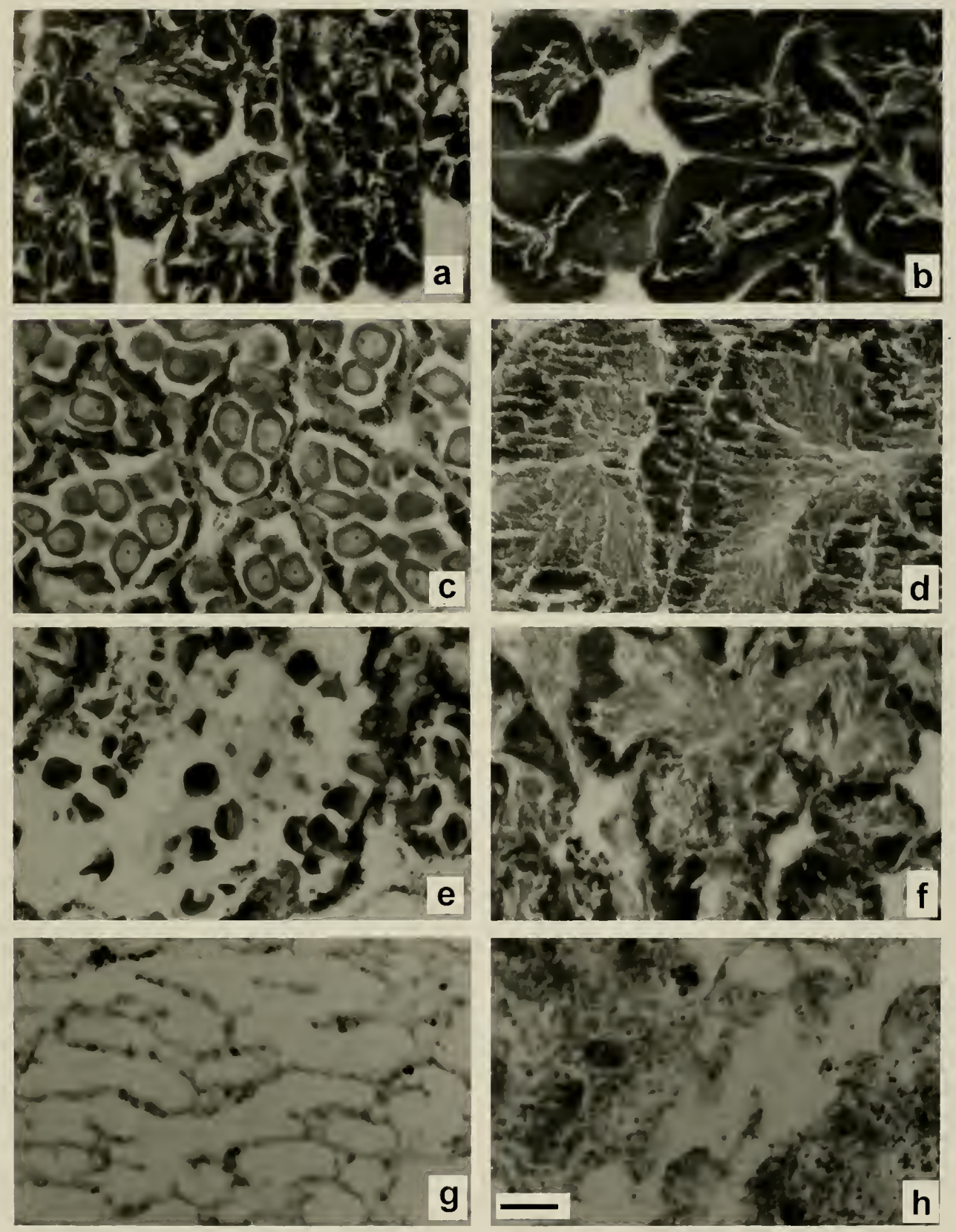

Figure 1. Photomicrographs of gonadal stages of *P. rugosa*. (a) Gonad classified as developing female; small oocytes growing attached to the follicle wall, male spent. (b) Developing male; thick layer of spermatocytes developing. (c) Mature female; large oocytes free in the lumen of follicles. (d) Mature male; large quantity of spermatozoa filling the follicles. (e) Partially spawned female; empty follicle with some residual oocytes. (f) Partially spawned male; a marked decrease in the number of spermatozoa filling the lumen. (g) Indifferent gonad; follicles with total absence of gametes. (h) Gonad spent; follicles collapsed, amebocytes phagocytizing residual gametes. Scale bar = 50 μm.

values obtained permit us to realize the correlation analysis of reproductive activity with temperature and photoperiod.

Data for photoperiod for this study were not determined directly by the authors. Data from nautical almanacs of the Secretaria de Marina of Mexico were used to define the photoperiod. The data correspond to the daily period of illumination, and an average in minutes of illumination was calculated for each month, between February 1993 and February 1994, for the latitude corresponding to Bahía Concepción.

A Spearman rank order correlation analysis was used to investigate the relationship between GI, temperature and photoperiod. Correlation analysis were carried out with the monthly values (n = 13).

RESULTS

A total of 311 specimens was collected, 33 females (10.6%), 55 males (17.7%), 65 hermaphrodites (20.9%), and 158 indifferentiated (50.8%). The range in shell length of pen shells was from 134 to 366 mm (258 mm average, 29 mm standard deviation).

In the hermaphrodite gonads, the development of both sexes was not synchronous. On the contrary, one sex was always in a more advanced stage of development (*i.e.*, the female phase was developing, whereas the male phase was spent) (Fig. 1a).

To describe the reproductive cycle, all of the organisms were considered, including the hermaphrodites. In the case of hermaphrodites, they were each considered as one individual accordingly with the more advanced developing stage. The similar range of gonadal development for small to large individuals indicated that all pen shell sampled were reproductively active. All five stages of gonadal development were observed (Fig. 1).

The reproductive cycle of *P. rugosa* from Bahía Concepción, Gulf of California, is summarized in Figure 2. Indifferent individuals were observed all year, except in June. In February 1993 and from December 1993 to February of 1994, most pen shell were indifferent staged (94.1%, 100%, 93%, and 100%, respectively). Gametogenesis commenced in March. Maturation was continuous through November. Ripe stage was present from April to November, except in September. The partially spawned stage was present in May and from July through November. Spent specimens occurred from May to September, except in June.

Monthly quantitative assessments of histological reproductive condition are illustrated in Figure 3a. From these data, it is apparent that the GI has a seasonal tendency along the year, with high values coinciding with ripe individuals and the fall of values coinciding with spawning activity. The values of GI were higher in April, June, and October and were lower from December to February. The GI values indicated that the gametogenesis started in March and continued until November, with pen shell quiescent from December to February.

Water temperature showed considerable seasonal variation (Fig. 3b) with extreme values of 31 °C in August and 19 °C in February.

The photoperiod (minutes of daily illumination) is illustrated in Figure 3c. The longest monthly average daily illumination in the study area occurred during May to July, with the highest in June (820 min). The minutes of daily illumination presented a decreased tendency during July through November. The shortest time of illumination occurred in November/December and January (640 min).

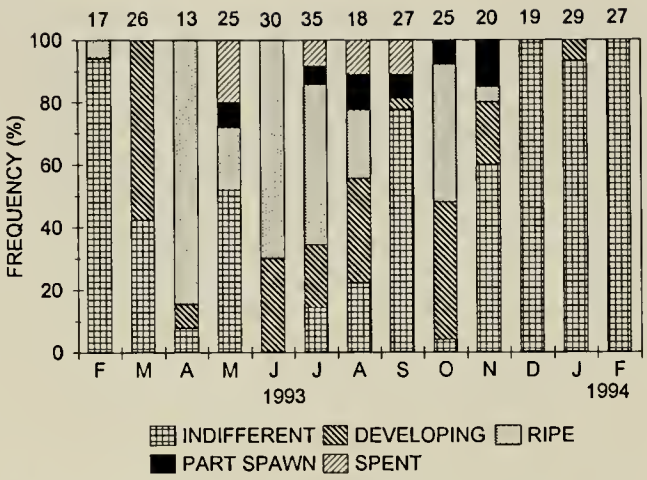

Figure 2. Reproductive cycle of *P. rugosa* from Bahía Concepción, Gulf of California, Mexico. Relative frequency of gonadal stages between February 1993 and February 1994. Observations of males and females are combined. Numbers at top indicate the sample sizes for each month.

In all cases significant correlation ($P < 0.05$) was found. The GI presented a positive correlation with temperature (n = 13; r = 0.85; P = 0.000192) and photoperiod (n = 13; r = 0.69; P = 0.008980). Temperature and photoperiod were positively correlated (n = 13; r = 0.59; P = 0.031929).

DISCUSSION

In Bahía Concepción, the rugose pen shell exhibits an annual gametogenic cycle, which commences in March with rapid prolif-

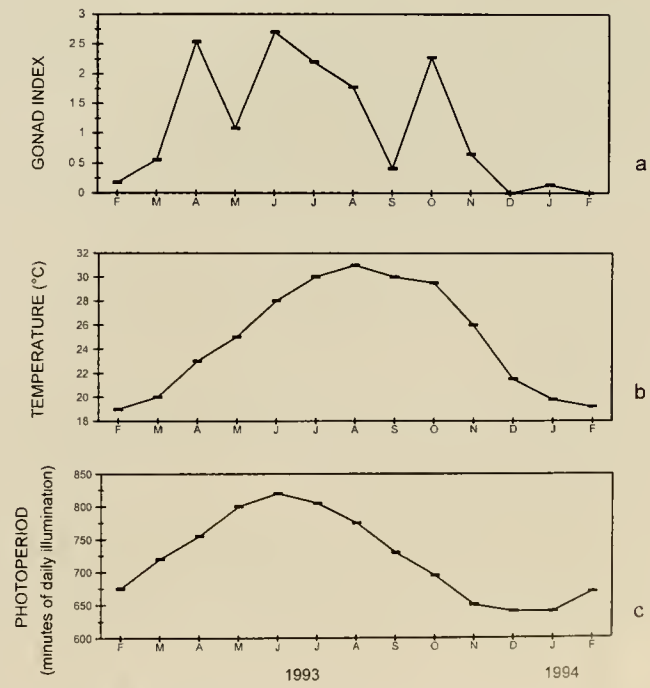

Figure 3. Monthly variation of GI (a), water temperature (b), and photoperiod (c) in Bahía Concepción, B.C.S., Mexico.

eration of gametes and ends by December. According to the histological analysis, the spawning occurs from May to November, except in June. Our results are in agreement with the reproductive cycle of *P. rugose* occurring in La Paz Bay, B.C.S., México, as described by Noguera and Gómez-Aguirre (1972), who documented that sexual maturation begins in mid-spring, with spawning occurring by late summer.

The characteristics of gametogenesis in *P. rugosa* from Bahía Concepción were similar to those described for *Spondylus leucacanthus* from Isla Danzante (Villalejo-Fuerte and García-Domínguez 1998). The gonad of *P. rugosa* has oocytes with the same degree of development, common for bivalves with a synchronic development. The histological examination additionally showed that *P. rugosa* is a hermaphrodite species; in this study 20.9% of pen shell presented this condition. Hermaphroditism is common in bivalves (Tranter 1958, García-Domínguez et al. 1996, Villalejo-Fuerte and García-Domínguez 1998).

There are two basic types of reproductive pattern exhibited by marine bivalves in the Gulf of California waters. Many bivalve species have no seasonal reproductive cycle, and their spawning activity is continuous, for example, *Megapitaria aurantiaca* (García-Domínguez et al. 1994) and *Pinctada mazatlanica* (García-Domínguez et al. 1996). Other bivalve species exhibit distinct seasonal reproductive cycles, such as *Dosinia ponderosa* (Arreola-Hernández 1997), *Chione undatella* (Baqueiro and Masso 1988), and *M. squalida* (Villalejo-Fuerte et al. 1996), which usually are related to temporal variations of environmental factors such as food availability, water temperature, and/or photoperiod.

The reproductive activity of *P. rugosa* was significantly correlated to the water temperature and photoperiod. The protracted period of reproductive activity (March to November 1993) of *P. rugosa* from Bahía Concepción coincides with the gradual increase of sea-surface temperature (from 20 °C until a maximum of 31 °C), and with increased values of photoperiod (720 min/day). The period of reproductive inactivity was clearly distinguished in winter (November 1993 to February 1994), and coincides with an abrupt decrease of 3.5 °C in the sea-surface temperature (26 °C) and with the photoperiod minimum values (640–650 min/day).

The Spearman correlation analyses indicated that the major environmental factor that directly influences the gonadal growth is the water temperature, suggesting that the production of gametes is stimulated by increases in temperature. The same has been observed for other bivalve species, such as *Spondylus leucacanthus* (Villalejo-Fuerte and García-Domínguez 1998) and *Argopecten circularis* (Villalejo-Fuerte and Ochoa-Baez 1993). However, in other bivalves from the Gulf of California, no clear relationship exists between gonadic development and water temperature (*e.g., M. aurantiaca* [García-Domínguez et al. 1994] and *P. mazatlanica* [García-Domínguez et al. 1996]). Although water temperature affects reproduction, other environmental factors may well play an integral role in determining the pattern of annual gonad activity for species in a given geographical area (Sastry 1970).

Giese and Pearse (1974) have reported photoperiod as a factor that influences spawning of invertebrates. However, it has not been widely studied in bivalves (Villalejo-Fuerte and Ochoa-Báez 1993). The temperature and photoperiod are positively correlated. But it may not be possible to separate the effects of these two factors with the data presented in this paper.

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CHROMOSOME SEGREGATION IN FERTILIZED EGGS FROM ZHIKONG SCALLOP *CHLAMYS FARRERI (JONES & PRESTON)* FOLLOWING POLAR BODY 1 INHIBITION WITH CYTOCHALASIN B

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ABSTRACT Chromosome segregation in fertilized eggs of the zhikong scallop, *Chlamys farreri*, following polar body 1 (PB1) inhibition with cytochalasin B (CB) was studied. The fertilized eggs were treated with CB (0.75 mg/L) at 7–10 min postfertilization until polar body 2 (PB2) was released in control groups. The embryos were sampled every 5–10 min after fertilization and fixed in Carnoy fixative. Chromosome segregation in both control groups and treated groups were analyzed using a hematoxylin stain method.

In fertilized eggs of control groups, the 19 tetrad chromosomes went through meiosis I and II, and released PB1 and PB2, finally reaching 19 chromatids. In CB treated groups, meiosis I proceeded normally and produced two groups of dyads, 19 in each group. With the CB treatment, both of the two dyad groups were retained in the eggs and entered meiosis II. The segregation in meiosis II had four patterns: bipolar, tripolar, tetrapolar, and unsynchronized segregation. When the two groups of dyads from meiosis I united, the treated eggs entered meiosis II through tripolar (40.9%) and bipolar (11.4%) segregation patterns. Otherwise the two groups of dyads segregated separately and formed tetrapolar segregation (15.7%). Also a small proportion of treated eggs (4.0%) underwent meiosis II in an "unsynchronized segregation" pattern, which means that the two groups of dyads from meiosis I did not segregate synchronously. There were 28.0% of treated eggs that could not be classified. The four segregation patterns produced different ploidies of embryos in CB treated groups, such as triploids, tetraploids, pentaploids, and aneuploids.

KEY WORDS: Zhikong scallop, *Chlamys farreri*, chromosome segregation, triploid, tetraploid, polar body

INTRODUCTION

Triploids can be induced by blocking the first polar body (PB1) in some mollusk species, such as American oyster, *Crassostrea virginica* (Stanley et al. 1981), Pacific oyster, *Crassostrea gigas (Thunberg)* (Quillet and Panelay 1986), Pacific abalone, *Haliotis discus hannai* (Arai et al. 1986), pearl oyster, *Pinctada martensii* (Jiang et al. 1987), and blue mussel, *Mytilus edulis* (Yamamoto and Sugawara 1988). Tetraploids were also reported among triploids in American oyster (Stanley et al. 1981) and in other mollusk species (Arai et al. 1986, Yamamoto and Sugawara 1988). Stephens and Downing (1988) reported that 91% tetraploid at 24-h postfertilization (PF) was produced by inhibiting PB1 in fertilized eggs from the Pacific oyster. In similar work, Guo et al. (1992a) reported that many aneuploids embryos (57.6%) were also produced. All of these results indicate that PB1 inhibition results in complicated chromosome segregation. Observation of chromosome segregation in the Pacific oyster explained the mechanism for formation of different ploidies when PB1 was blocked (Guo et al. 1992b). Three different types of segregation, including "tripolar segregation," "united bipolar segregation," and "separated bipolar segregation" were evident. Later, the chromosome segregation in triploid Pacific oysters was also studied when eggs from triploids were fertilized with diploid sperm and PB1 was blocked with CB (Que et al. 1997). The observation showed that there were also three types of segregation patterns, confirming the mechanism by which viable tetraploid Pacific oysters can be successfully induced through blocking PB1 in fertilized eggs from triploids (Guo and Allen 1994).

In the zhikong scallop, *Chlamys farreri*, blocking PB1 in fertilized eggs from normal diploids can result in triploid, tetraploid, pentaploid, and aneuploid embryos. We have also found that both triploids and tetraploids can survive to 2–3 mm juvenile stage (unpublished). In this paper, the behavior of chromosome segregation was observed in fertilized eggs from normal diploids when PB1 was blocked with CB, offering an explanation for the formation of embryos with different ploidies.

MATERIALS AND METHODS

Gametes

Parent scallops were from Rizhao and Qingdao, Shandong, China. The scallops were conditioned indoors to accelerate gonad maturity. Gametes were obtained through natural spawning. Eggs were collected with a 25-μ screen and resuspended into 2 ~ 3 L seawater at 20 °C, ready for fertilization. Sperm were prepared by screening sperm suspension through a 25-μ nylon screen. For fertilization, sperm were added to the egg suspension at a final density of 5–7 sperm per egg. Fertilization, treatment, and embryo culture were all conducted at 20 °C.

Treatment and Sampling

PB1 in fertilized eggs was blocked with 0.75 μg/mL CB dissolved in dimethyl sulfoxide (DMSO-final concentration 0.1%). CB treatment began at 7–10 min PF and ended when the second polar body (PB2) in control groups was observed under microscope. Fertilized eggs in both control groups and CB-treated groups were sampled every 5–10 min during development until 75 min PF. Samples were directly fixed in Carnoy fixative (methanol: acetic acid = 3:1), which was changed twice, and the samples were then stored at 4 °C before analysis. The experiment was repeated four times using different parent scallops.

Chromosome Observation

Slides for observing chromosomes were made by a modified squashing method. The staining solution was made by dissolving 0.5% hematoxylin in 45% acetic acid, with ammonium iron sulfate

dodecahydtate as a mordant (about 0.5%). Embryo samples were dropped and spread on clean slides. Excessive fixative was allowed to run off the slides, and then drops of staining solution were added onto the samples just before the fixative evaporated. A clean cover glass was placed gently on the samples. Before squashing on filter paper, slides were warmed slightly by passing them across an alcohol burner. Then, the cover glass was sealed on all four sides. Alternatively, the whole cover glass was sealed with neutral balsam after removal by icing the slides.

Slides were examined with a Nikon compound microscope. Photographs were taken using LUCKY black and white film (ASA 100 and 400).

RESULTS

Initially, normal diploid eggs were observed in prophase of meiosis I (Fig. 1a). Zhikong scallop has a diploid number of 38 chromosomes (Wang et al. 1990). Nineteen tetrads were observed in the unfertilized eggs.

In control groups, the 19 tetrads began to segregate at about 9–10 min PF, then the tetrads in the majority of fertilized eggs segregated into 38 dyads, and then divided into two groups, 19 in each group (Fig. 1b). Later, one group of dyads condensed and released as PB1 (Fig. 1c). The remaining 19 dyads continued meiosis II and segregated into two groups of chromatids (Fig. 1d). One of the groups of chromatids was released as PB2 in most fertilized eggs at 40–43 min PF. Normally, the two polar bodies were positioned next to each other (Fig. 1e). As for the chromatids from sperm, at first, they could only be observed as dark-stained material. Only during mitosis 1, did the chromatids from egg and sperm unite, yielding 38 chromosomes.

In CB-treated groups, chromosome segregation was complicated. After fertilization, the 19 tetrads segregated into 38 dyads (Fig. 1g). Under the microscope, no PB1 was released in the majority of treated, fertilized eggs during CB treatment. Thus, 38 dyads in fertilized eggs entered meiosis II, and chromosome segregation differed greatly from that in control groups. Four patterns of segregation were observed: bipolar, tripolar, tetrapolar, and unsynchronized. Some segregations could not be classified. When the 19 dyads from PB1 united with the other 19 dyads, the chromosome segregation in meiosis II proceeded with bipolar or tripolar segregation.

Bipolar Segregation

The 19 dyads from PB1 united with the remained 19 dyads, and went through meiosis II together (Fig. 1h). All 38 dyads segregated in a bipolar pattern just like normal meiosis II, and divided into two groups of sister chromatids, 38 in each group (Fig. 1i). One of the two groups of chromatids was released as PB2 after CB treatment. This pattern of chromosome segregation could result in triploids.

Tripolar Segregation

The 38 united dyads divided into three groups, apparently at random (Fig. 1j), and the dyads in each group segregated in two directions. Finally, the chromatids migrating in one direction united with the chromatids from its neighboring group at a pole, forming three groups of chromatids (Fig. 1k). The number of chromatids in the three groups varied considerably, apparently depending upon random distribution of dyads before meiosis II. Rarely, one of the three groups had exactly 19 chromatids. In this pattern of chromosome segregation, the three groups of chromatids had probability of being released as PB2 after CB treatment.

Sometimes the 19 dyads from the unreleased PB1 fail to unite with the remaining 19 dyads in the fertilized eggs (Fig. 1l). They entered meiosis II independently, resulting in two patterns of chromosome segregation: tetrapolar and unsynchronized segregations.

Tetrapolar

In both dyads groups, the chromosomes segregated in a normal bipolar pattern. The final result was that four groups of chromatids formed, 19 chromotids in each group (Fig. 1m).

Unsynchronized Segregation

The unreleased dyads and the remaining dyads in the fertilized egg went through meiosis II asynchronously. Sometimes one group of dyads did not go through meiosis II, but remained unchanged, and another group of dyads went through meiosis II and underwent bipolar segregation to anaphase (Fig. 1n).

Unclassified

In addition to the above segregation patterns, there were also other patterns that could not be classified. In fertilized eggs with 38 united dyads, only some began meiosis II segregation; whereas, some were left as dyads. In some treated fertilized eggs, the 38 dyads went through meiosis II, but the 76 chromatids distributed themselves randomly. No segregation poles were observed (Fig. 1o).

The frequencies of the four segregation patterns were calculated from embryos where PB1 had been blocked (Table 1). On average, the majority of treated eggs went through meiosis II as tripolar segregations (40.9%), 11.4% were bipolar, and 15.7% were tetrapolar. Only a small proportion of treated eggs (4.0%) went through unsynchronized segregation. Finally, 28.0% of segregations in treated eggs could not be classified.

DISCUSSION

We made slides for observing chromosome segregation by a modified squashing method using a hematoxylin stain. For fertilized eggs and embryos from the zhikong scallop, this procedure was quite useful. In fertilized eggs of the Pacific oyster, orcein dissolved in 60% acetic acid employed chromosome observations (Guo et al. 1992a, Que et al. 1997). In the zhikong scallop, we have tried orcein staining, but it produced poor contrast. Hematoxylin is a typical chromosome stain (Sharma and Sharma 1980). Normally hematoxylin solution must be made in advance to ripen for a few weeks. In this experiment, the stain solution was modified as 0.5% dissolved in 45% acetic acid with ammonium iron sulfate as mordant and could be used instantly. In addition, this method produced satisfactory results for observing chromosomes in the fertilized eggs and embryos from the jinjiang oyster, *Crassostrea ariakensis* (unpublished). We suggest this new staining method for chromosome observations in bivalve mollusk.

Chromosome Segregation

Unfertilized eggs of normal diploid zhikong scallops are arrested at late prophase of meiosis I. Only after fertilization, did the eggs continue meiosis I and II, releasing PB1 and PB2. In the end, 19 maternal chomatids remained in the fertilized eggs. This pattern of chromosome segregation is common among bivalve mollusks (Longo and Anderson 1969).

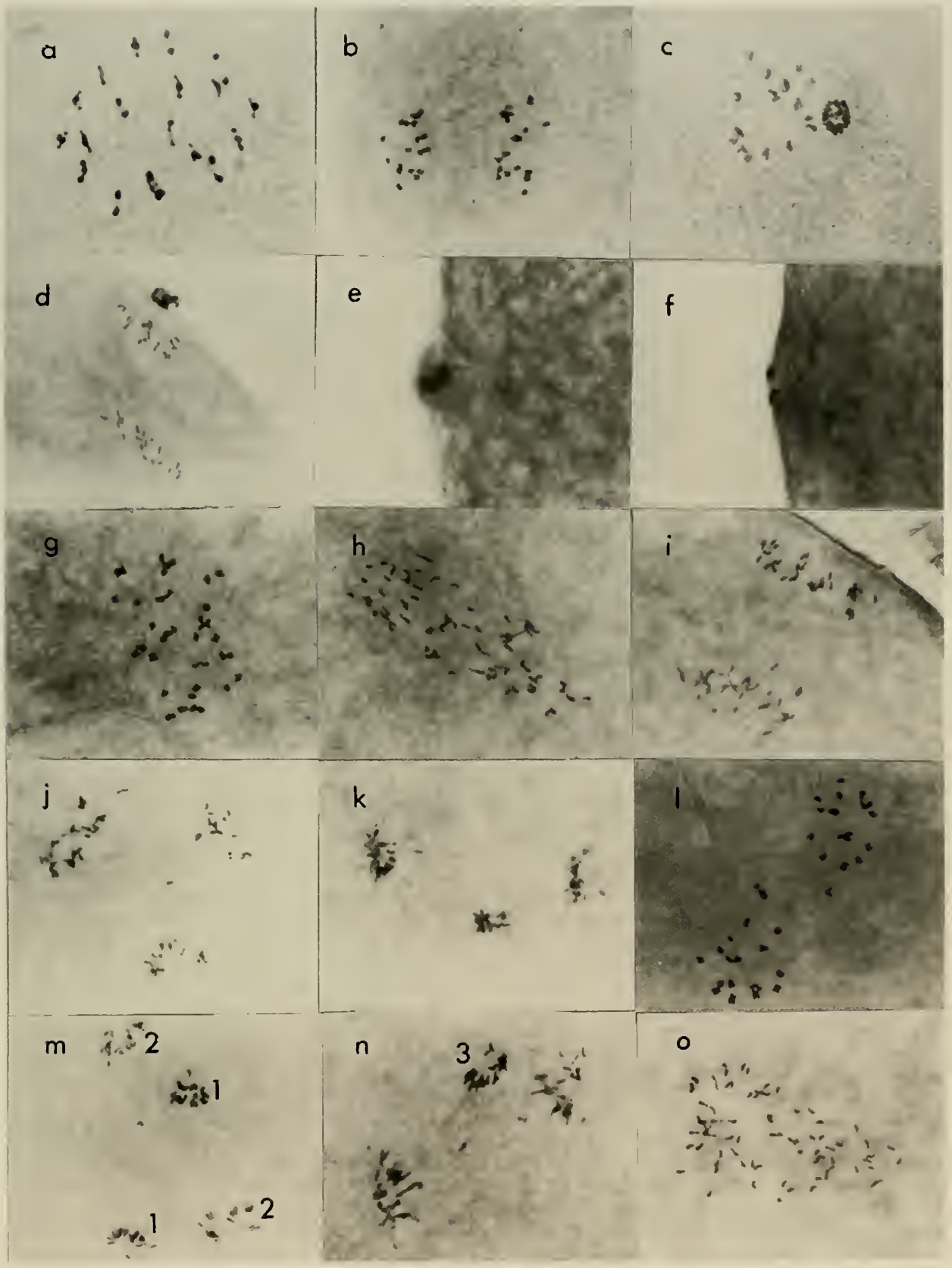

Figure 1. Segregation patterns observed in fertilized eggs from diploid zhikong scallop, *Chlamys farreri*, following normal fertilization (a–e) and PB1 blocking with Cytochalasin B (f–o). a–e: meiosis in normal fertilized eggs; f: two polar bodies positioned side-by-side in fertilized eggs following PB1 blocking with CB; g: the united 38 dyads. h–i: bipolar segregation pattern; j–k: tripolar segregation pattern; l–m: tetrapolar segregation pattern. 1 and 2 indicated two separate poles; n: unsynchronized segregation pattern. 3 indicated dyad groups; o: unclassified segregation pattern.

TABLE 1.

Chromosome segregation patterns (%) in fertilized eggs when PB1 was blocked with CB in zhikong scallop *Chlamys farreri*.

Replicate (Number)	Chromosome Segregation Patterns					
	n	Bipolar	Tripolar	Tetrapolar	Unsynchronized	Unclassified
1	109	11.0	45.9	17.4	1.8	23.9
2	162	7.4	46.9	17.3	4.3	24.1
3	106	13.2	46.2	11.4	2.8	26.4
4	85	14.1	24.7	16.5	7.1	37.6
Average		11.4	40.9	15.7	4.0	28.0

In CB-treated groups, chromosomes in fertilized eggs, following PB1 blocking, segregated in four patterns: bipolar (11.4%), tripolar (40.9%), tetrapolar (15.7%), and unsynchronized (4.0%). Bipolar, tripolar, and tetrapolar segregation patterns were similar to those reported in diploid (Guo et al. 1992b) and triploid Pacific oysters (Que et al. 1997) when PB1 was blocked.

In addition, in the zhikong scallop a small proportion of treated eggs (4.0%) went through meiosis II asynchronously. Blocked dyads from PB1 failed to unite and segregated asynchronously from the remaining dyads. Sometimes the remaining dyads went to anaphase of meiosis II and divided into two groups of chromatids, while the dyads from blocked PB1 remained paired and skipped meiosis II, leaving three chromatin groups. In eggs of triploid Pacific oysters, asynchronous segregation was also observed when crossed with a normal sperm of diploid followed by PB1 inhibition by CB (Que et al. 1997). In the Japanese pearl oyster, *Pinctada fucata martensii*, Komaru et al. (1990) reported that three groups (20.6%) and four groups (17.6%) of maternal chromatin were produced by blocking PB1. The observation of three groups of chromatin might be explained in two ways: asynchronous segregation or tripolar chromosome segregation, both resulting in three chromatin groups. The percentage of fertilized eggs with three groups of maternal chromatin (20.6%) as observed by Komaru was much lower than our observations of tripolar segregation (40.9%) and asynchronous segregation (4.0%) in the zhikong scallop. This is possibly caused by differences in chromosome segregation between the different species or because the conditions of CB treatment were different. In diploid Pacific oysters, chromosome segregation following PB1 inhibition was observed to pass through meiosis II synchronously (Guo et al. 1992b).

In addition to the described four segregation patterns, there was a large proportion of chromosome segregations (28.0%) that could not be classified, such as 76 chromatids scattered randomly. Unclassified segregation patterns have also been observed in both diploid and triploid Pacific oysters (Guo et al. 1992b, Que et al. 1997). Considering the results of this experiment and those in diploid and triploid Pacific oysters and pearl oysters, we suggest that tripolar, tetrapolar, bipolar, and unsynchronized segregation patterns are the normal ways for fertilized eggs to go through meiosis II after PB1 blocking.

Observations of chromosome segregation using the squashing method provide an incomplete picture of cytological events, because compression of the eggs transforms the three dimensionality of the meiotic plates into a plane, thus rearranging the position of chromosomes. This method also fails to display centrosomes and spindles that play an important role in meiosis. Observing centrosomes and spindles might provide a clearer picture of how chromosomes segregated. Especially for the centrosome, its number and replication are critical factors in the chromosome segregation. Normally the centrosome from sperm does not participate in meiosis (Sluder et al. 1993), and the centrosome from maternal replicates two times with each meiotic stage, resulting in the normal bipolar segregation. In this experiment, we hypothesize that centrosome number is the primary factor controlling patterns of chromosome segregation. With PB1 blocked in eggs, centrosome number could change profoundly, affecting chromosome segregation in meiosis II. Centrosome numbers could range from 2–4, depending upon whether centrosomes replicated, and could result in bipolar, tripolar, or tetrapolar chromosome segregation patterns. This supposition must be tested by visualization of the centrosomes, spindles, or both.

Ploidy Consequences

In the zhikong scallop, diploid, triploid, tetraploid, pentaploid, and aneuploid 2–4 cell stage embryos were all produced when PB1 was blocked in fertilized eggs. Both triploid and tetraploid zhikong scallops survived to juvenile stage (21.3% triploid and 1.9% tetraploid in one group, unpublished). The various ploidy consequences of PB1 blocking relate to the different chromosome patterns, as observed in this study.

First, bipolar segregation patterns formed two groups of 38 chromatids. Either of the two chromatids group could be released as PB2, leaving 38 chromatids. No matter which group was released, triploids would be produced by bipolar segregation with 19 chromosomes contributed by the sperm.

For tetrapolar segregation patterns, four separated chromatids groups were formed after meiosis II, 19 chromatids in each group. The ploidy consequences would depend upon how many chromatid groups would be released with PB2. Release of one group would produce tetraploids; whereas, release of two groups would produce triploids and release of three would produce diploids. After CB was washed off, embryo development showed that some fertilized eggs in the treated groups released one PB, and some fertilized eggs released two PBs positioned side-by-side (Fig. 1f) or separated from each other on the egg. Rarely were these two polar bodies positioned next to each other, as in Figure 1e. It was impossible by our methods to observe total number of chromatids in released PBs. This problem might be resolved by using special staining methods to label chromatids individually, such as *in situ* fluorescent hybridization.

The ploidy consequences of embryos after tripolar segregation were the most complicated because of random allocation of chromatids at three poles and the random release of PB2. The metaphase and anaphase period in meiosis II were very short, so it was not practical to count numbers of chromatids at the three poles in most fertilized eggs. By counting the chromosome of 2–4 cell embryos, we could infer that chromosome number varied highly.

In tripolar segregations, tetraploids would be produced only when one pole had exactly 19 chromatids, and the chromatids at this pole were released as PB2. If the 19 chromatids at one pole remained in the eggs, and the chromatids at the other two poles were released as PB2, diploids would be produced. Otherwise, aneuploids resulted. The majority of fertilized eggs proceeded by tripolar segregation (40.9%), explaining why about 23.3% of 2–4 cell stage embryos were aneuploid (unpublished). In most aneuploids, chromosome numbers were distributed mainly into three groups: 42–48, 62–69, or 83–89, most likely the result of random allocation of chromatin from the three poles.

Unsynchronized segregation resulted in three groups of chromatin, two with 19 chromatids in each and the other with 19 dyads from blocked PB1. Diploids, triploids, and tetraploids could possibly be produced, depending upon which group was released as PB2. Supposing one group of 19 chromatids was released as PB2, tetraploids would be produced. If 19 dyads were released as PB2, triploids would be produced. If two groups of chromatin were released as PB2, triploids or diploids would be produced.

Pentaploids were also observed at the 2–4 cell embryo stage (unpublished). The formation of pentaploidy was probably caused by the failure of PB2 to be released in fertilized eggs after PB1 was blocked with CB. Thus, the 76 chromatids from maternal chromotids plus the 19 chromatids from sperm formed pentaploids.

Clearly, differences in chromosome segregation resulted in different ploidy consequences and agree with the proposed mechanism to form different ploidies in diploid Pacific oyster when PB1 was blocked (Guo et al. 1992b). PB1 blocking is also an effective way to induce triploids and tetraploids. Both triploid and tetraploid embryos have been produced through blocking PB1 in fertilized eggs from normal diploid, such as in Pacific oyster (Guo et al. 1992a), American oyster (Stanley et al. 1981), Pacific abalone (Arai et al. 1986) and blue mussel (Yamamoto and Sugawara 1988).

In summary, the inhibition of PB1 in fertilized eggs of zhikong scallop with CB resulted in complicated chromosome segregation patterns, including bipolar, tripolar, tetrapolar, unsynchronized, and unclassified segregations, producing diploid, triploid, tetraploid, pentaploidy, and aneuploid embryos. This study provided cytological evidence about possible formation of different ploidies and valuable information on polyploid induction.

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REPRODUCTIVE CYCLE OF *ARGOPECTEN VENTRICOSUS* (SOWERBY 1842) (BIVALVIA: PECTINIDAE) IN THE RADA DEL PUERTO DE PICHILINGUE, B.C.S., MEXICO AND ITS RELATION TO TEMPERATURE, SALINITY, AND FOOD

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ABSTRACT The reproductive cycle of the catarina scallop *Argopecten ventricosus* and its relation to temperature, salinity, and quantity of food was studied in the Rada del Puerto de Pichilingue, B.C.S. Mexico, from April 1995 to March 1996. Organisms were obtained from a hatchery and grown on the bottom. Ripe organisms occurred throughout the year showing the lack of seasonality in its reproduction. No consistent relation between reproductive cycle and environmental factors or food was evident. The muscle yield index showed a significant positive correlation with temperature, but it had no correlation with gonadosomatic index. The relation between the muscle yield index and seston with the reproductive cycle suggested the transference of energy from the muscle to the gonad and directly from the seston ingested. This relation suggested that *A. ventricosus* is a conservative and opportunistic species depending on the available food. Histochemical analysis revealed the transference of carbohydrates from the intestinal loop to the gonad and therefore to the oocytes.

KEY WORDS: *Argopecten*, reproductive cycle, bivalves, histochemistry, seston, food index

INTRODUCTION

The scallop *Argopecten ventricosus* (Sowerby 1842) is distributed from Isla Cedros and the Gulf of California to Peru (Keen 1971). *A. ventricosus* supports an important fishery in northwest Mexico, especially in Baja California Sur (Chávez-Villalba and Cáceres-Martínez 1992). It is an important resource because of the high commercial value of its adductor muscle (Villalejo-Fuerte and Ochoa-Báez 1993).

The necessity of measures for the regulation of the fishery has prompted several studies about reproduction of the catarina scallop in Baja California Sur (Baqueiro et al. 1981, Cáceres-Martínez et al. 1990, Villalejo-Fuerte and Ochoa-Báez 1993, Félix-Pico et al. 1995).

The reproductive cycles of scallops are influenced by changes in environmental variables, such as temperature and food (MacDonald and Thompson 1985, Barber and Blake 1991), and by genetic characteristics (Barber and Blake 1991). Gametogenesis needs a lot of energy (Sastry 1979). This energy is obtained directly from the seston or from storage organs or tissues, like the digestive gland, mantle, and adductor muscle (Ansell 1974, Gabbott 1975, Barber and Blake 1983).

The seston includes live plankton, organic detritus, and inorganic particles (Navarro and Thompson 1995). The quantity and quality of seston varies in response to physical and biological factors such as tides, storms, wind, bacteria, fungi, and primary consumers (Berg and Newell 1986, Mann 1988).

The objective of the study is to examine the reproductive cycle of *A. ventricosus* in relation to its condition, histochemical composition of somatic and reproductive tissues, temperature, salinity, and quantity of available food.

MATERIALS AND METHODS

Between April 1995 and March 1996, 30 specimens of *A. ventricosus* (shell height mean ± SD = 5.25 ± 0.02 cm) were collected randomly per month by diving between 3- and 4-m depth from a population grown in the Rada del Puerto de Pichilingue, B.C.S., Mexico (24°16′N; 110°19′W). These organisms were initially produced in September 1994 at our hatchery at Universidad Autónoma de Baja California Sur and seeded on the bottom in February 1995. The surface water temperature and salinity were recorded at the time of sampling. Total soft body, adductor muscle, and gonad wet weights were recorded for each specimen.

Reproductive Cycle

The scallops were fixed in 10% formalin. Tissue sections were taken through the middle of the gonad, dehydrated in alcohol, and embedded in paraffin wax. Sections (5 μm) were placed on slides and stained with hematoxylin-eosin (Humason 1979). Gametogenesis (either spermatogenesis or oogenesis) of *A. ventricosus* was divided into five stages (undifferentiated, developing, ripe, spawning, and spent) on the basis of the developmental stages defined by Villalejo-Fuerte and Ochoa-Báez (1993) for the same species and our own observations.

Undifferentiated Stage

Abundant connective tissue, without germ cells or residual gametes. It was not possible to distinguish the sex.

Developing Stage

In the female, this stage is characterized by the presence of variable quantities of developing oocytes attached to the follicle

wall. Some detached ripe oocytes occurred in the lumen of the follicle. In the male, this stage had variable quantities of germinal cells, spermatocytes, spermatids, and ripe spermatozoa. Interfollicular connective tissue decreases and follicles increase in area as the result of the accumulation of ripe gametes.

Ripe Stage

In the female, there were abundant, ripe polygonal-shaped oocytes free within the follicles. Yolk droplets were observed in the oocyte cytoplasm. Some developing oocytes remained attached to the follicle wall by a slender stalk. In the male, this stage was characterized by follicles filled with ripe spermatozoa arranged in characteristic radial bands with tails pointing toward the center of the lumen. Almost all the connective tissue has been completely replaced by follicles forming the gonadic tissue, which is occupied by gametes.

Spawning Stage

The walls of follicles become broken. Variable quantities of unspawned oocytes and spermatozoa were observed into the follicles. Free spaces inside the follicles were abundant. Some follicles are completely devoid of gametes.

Spent Stage

The follicles were empty, with the exception of some residual oocytes and spermatozoa. Connective tissue begins increasing. The broken follicles are invaded by phagocytes. The relative frequencies of the stages of gonadal development throughout the year were obtained. This enabled the description of the reproductive cycle.

Gonadosomatic Index (GSI)

This index in wet weight was calculated according to Sastry and Blake (1971).

$$GSI = \frac{GW}{TSBW} * 100$$

Where GSI is the gonadosomatic index, GW is the gonad weight in grams, and TSBW is the total soft body weight in grams.

Muscle Yield Index (MYI)

The muscle yield index was calculated as an indicator of the condition of the scallops (Cáceres-Martínez et al. 1990).

$$MYI = \frac{MW}{TSBW} * 100$$

Where MYI is the muscle yield index, MW is the weight of muscle in grams, and TSBW is the total weight of the soft body in grams.

Histochemical Analysis

Four scallops corresponding to each stage of gonadal development (twenty in total) were collected in September 1995 for histochemical analysis (qualitative analysis) of gonad, mantle, and muscle to determine carbohydrate and lipid content. Unfortunately, we did not take samples since April 1995 to get an annual cycle. Tissue sections were embedded in paraffin wax and O.C.T. compound (an embedding medium for frozen tissue specimens). Sections 5-μm thick from paraffin wax and sections 16-μm thick from O.C.T. were placed on slides. The oil red technique (Spannhof 1966, Martoja and Person 1970) was used on frozen cuts to determine unsaturated lipids. Periodic acid of the Schiff-Malt technique was used to determine glycogen (Martoja and Person 1970, Sheehan and Hrapchak 1973, Humason 1979), and the blue alzian technique was used to detect acid mucopolysaccharides (Spanhoff 1966, Martoja and Person 1970).

Seston Analysis

During the study period, every 15 days, 12-L of unfiltered seawater samples of the scallop-sampling area were collected in clean plastic containers and transported to the laboratory. The seawater samples were collected at 3.5-m depth, close (about 15 cm) to the sandy bottom on which the scallops grew. The water was screened through a 180-μm Nitex mesh to eliminate large zooplankton and debris before analysis.

For dry weight and chemical analysis, 2-L of seawater for each filter (six filters in total every 15 days) were immediately filtered under gentle vacuum through washed, precombusted, preweighed Whatman GF/C filters, 4.7-cm diameter. Three filters for chemical analysis were stored at −40 °C until the analysis was done. Three filters for dry weight were dried in an oven at 80 °C for 24 h. Then they were weighed and combusted at 475 °C for 4 h. Finally, filters were reweighed after cooling in a desiccator. The particulate organic matter (organic seston) was obtained by difference of both weights.

For chemical analysis, two filters per month (one filter per sampling) with 2-mL of distilled water were ground at 5 °C in an ice bath. A 400-μL aliquot was used for lipid determination using the Bligh and Dyer (1959) method. Carbohydrates were analyzed in a 300-μL sample by the method of Dubois et al. (1956), modified by Malara and Charra (1972a). Proteins were analyzed in a 300-μL aliquot by the method of Lowry et al. (1951), modified by Malara and Charra (1972b). Results of chemical analysis were standardized for volume of seawater filtered.

Total Seston (TS)

The TS was obtained as the sum of inorganic seston and organic seston (dry weight).

Inorganic Seston/Organic Seston Ratio (IS/OS ratio)

This ratio was obtained to relate (monthly) inorganic seston to organic seston.

Food Index (FI)

An evaluation of the nutritional value of the seston throughout the annual cycle in the Rada del Puerto de Pichilingue was done using the 3 major biochemical components of the seston (lipid, carbohydrate, and protein). Thus food quantity was defined as the sum of these components and a food index was calculated according to Widdows et al. (1979) as the percentage of food material contained in the total seston.

$$FI = \frac{F}{TS} * 100$$

Where FI is the food index, F is the food material (mg/L), and TS is the total seston (mg/L).

RESULTS

Reproductive Cycle

The scallop *A. ventricosus* is a functional hermaphrodite. In the female and male follicles, the gametes were in the same developmental stage. The gonad showed well-differentiated male and female areas. Figure 1 summarizes the reproductive cycle of *A. ventricosus*. The presence of ripe gonads throughout the year indicated a prolonged reproductive period with a lack of a clear seasonal pattern. Despite this, there was a major resting period in June and September 1995 where the undifferentiated stage reached a maximum (91.3 and 46.15%, respectively). The spawning stage was observed in 9 of the twelve months sampled but reached the maximum value in August 1995 (50%).

Environmental Parameters

Temperature and salinity fluctuated relatively little (Fig. 2a). The maximum water temperature was in September 1995 (29.5 °C), and the minimum (20.5 °C) in January 1996. The maximum salinity was in January, February, and March (37 ‰), and the minimum in August and September 1995 (34 ‰).

Gonadosomatic Index

The GSI supported the results obtained in the histological analysis (Fig. 2b). The values were at a minimum in April, May, June, September, January, and increased drastically from January (4.08%) to February (7.79%) and March (8.75%).

Muscle Yield Index

The MYI was at a maximum in June and September 1995 (45.8 and 46.9%, respectively) and was at a minimum in April 1995 (33.7%) and from December 1995 to March 1996 (Fig. 2c). The MYI showed a significant positive correlation with temperature ($r = 0.797$; $P = 0.001$; $n = 12$), and a significant negative correlation with salinity ($r = -0.788$; $P = 0.002$; $n = 12$). With the GSI, there was no significant correlation ($r = -0.405$; $P = 0.190$; $n = 12$).

Histochemical Analysis

The results of the histochemical analysis of gonad, adductor muscle, and mantle are in Table 1. Positive results were found for glycogen in the female area of the gonad (developing and ripe oocytes) and in muscle fibers. Unsaturated lipids were found in the female area of the gonad (developing and ripe oocytes). Acid mucopolysaccharides were found in the interfollicular connective tissue of developing stage gonads (male and female areas), epithelium and food content of intestinal loop, and in the epithelium and connective tissue of mantle.

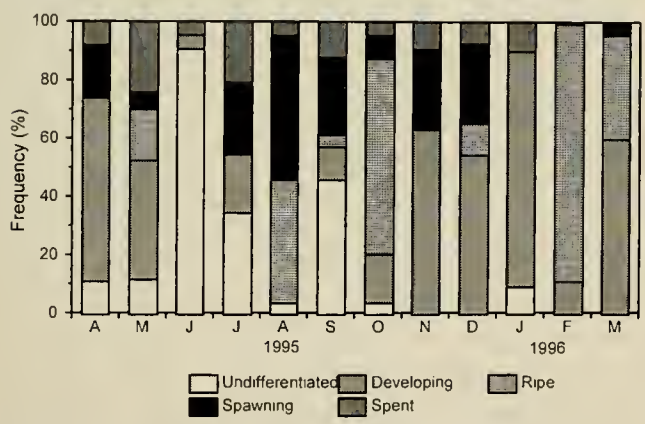

Figure 1. Reproductive stages of *Argopecten ventricosus* in the Rada del Puerto de Pichilingue, B.C.S., Mexico (n = 30).

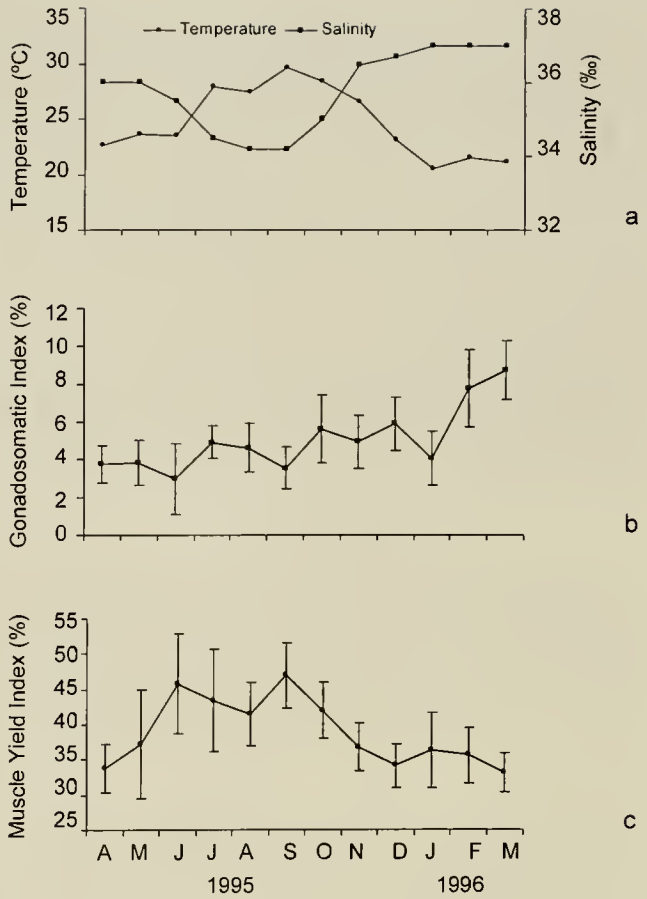

Figure 2. Water temperature and salinity in the Rada del Puerto de Pichilingue, B.C.S., Mexico (a) and gonadosomatic (b) and muscle yield index (c) of *Argopecten ventricosus*. (Error bars = SD).

Total Seston, Inorganic Seston, and Organic Seston

Sediment resuspension was caused mainly as a consequence of tidal currents and wind. In this area, maximum values of TS and IS were found in April, July, December, and January. Higher values of OS were found in April, December, and January (Fig. 3a).

Inorganic Seston/Organic Seston Ratio

The IS/OS ratio had no clear relation with the reproductive cycle (Fig. 3a). However, it showed a significant positive correlation with salinity ($r = 0.676$; $P = 0.015$; $n = 12$).

Food index

The FI expresses the quality of the diet available to a filter-feeding organism. This FI showed maximum values in July, September, and November 1995 (4.99, 5.03, and 4.97%, respectively) and minimum values in April-May 1995 (2.79 and 2.92%, respectively) and February 1996 (2.78%) (Fig. 3b). It did not show a clear relation with the reproductive cycle.

TABLE 1.

Histochemical tests performed on gonad, muscle, and mantle of *Argopecten ventricosus*.

Substance Tested	Technique	Control	Gonad	Muscle	Mantle
Glycogen	Pas-Malt	Rat liver	– oo ++ ro ++ do	++	–
Unsaturated lipids	Oil Red	None	– oo ++ ro ++ do	–	–
Acid mucopolysaccharides	Alzian Blue	None	++ ifd + ifr	–	+ mec

Abbreviations: –, not detected; +, positive reaction; ++, strong positive reaction; do, developing oocytes; ifd, interfollicular connective tissue of developing stage gonads (male and female area), epithelium and food content of intestinal loop; ifr, interfollicular connective tissue of ripe stage gonads; mec, mantle epithelium and connective tissue; oo, oogonias; ro, ripe oocytes.

DISCUSSION

The cytological characteristics of the gonad of *A. ventricosus* in the Rada del Puerto de Pichilingue, B.C.S. were similar to those described by Villalejo-Fuerte and Ochoa-Báez (1993) for the same species in Bahía Concepción, B.C.S., and for other pectinids, like *Patinopecten yessoensis* (Motavkine and Varaksine 1983) and *Placopecten magellanicus* (Beninger 1987). Male and female follicles developed simultaneously and the gametes were spawned at about the same time.

The gonads of *A. ventricosus* contained gametes in different stages of development in all the months during the annual cycle, although in lesser amount in June when the majority of the specimens were in the undifferentiated stage. Ripe organisms were present throughout the year, which suggests that this species reproduces throughout the year. Similarly, the presence of ripe organisms of *A. ventricosus* all year has been reported in other locations of Baja California Sur (Baqueiro et al. 1981, Félix-Pico et al. 1995).

Although the temperature is an important environmental factor in the regulation of bivalve reproduction (Sastry 1979), in this work, neither temperature nor salinity showed a clear relation with the reproductive cycle of *A. ventricosus* in the Rada del Puerto de Pichilingue because partly spawning scallops appear throughout the annual cycle. Maximum and minimum water temperatures coincided with the spawning (histologicaly detected) of August-September and December, as did the minimum and maximum values of salinity. The above suggests that the changes in temperature and salinity may be responsible for triggering spawning, but did not affect directly the gonadal maturation process.

In this work, the MYI did not show a significant negative correlation with GSI, but reproductive activity was present year around. An explanation of this unclear relation of the MYI with the reproductive activity is that in the Rada del Puerto de Pichilingue this species uses the available food in the environment more than muscle reserves for the gonadal maturation when the food is abundant, and they use the muscle reserves when the food abundance is poor. A transference of energy from the muscle to the gonad in *A. ventricosus* had been suggested by Cáceres-Martínez et al. (1990) and Villalejo-Fuerte and Ceballos-Vázquez (1996).

Bayne (1976) divided the bivalves into two groups based on their gametogenic pattern: 1) "conservative" species where gametogenesis occurs from energy stored in the tissue, and 2) "opportunistic" species where gametogenesis occurs when there was abundant phytoplankton. In this case, *A. ventricosus* would be named both opportunistic and conservative depending on the available food.

The MYI had a positive correlation with temperature. This may be the environmental variable that influences the transference of stored reserves from the adductor muscle to the gonad of *A. ventricosus*, as happens in *A. irradians* (Sastry and Blake 1971, Barber and Blake 1981, MacDonald and Bourne 1987). For salinity, a negative correlation with MYI was observed but the influence of salinity in the transference of nutrients remained unclear.

Le Pennec and Beninger (1991) observed that through most of the energy supplied to the developing gametes comes from protein and glycogen reserves in the adductor muscle, there is also energy transference from the reabsorption of residual oocytes and from the transference of nutrients from the intestinal loop to the gonad. The intestinal loop penetrates into the gonad and has a digestive function (epithelium with intracellular and extracellular digestion) and there is a direct transference of the metabolites from the in-

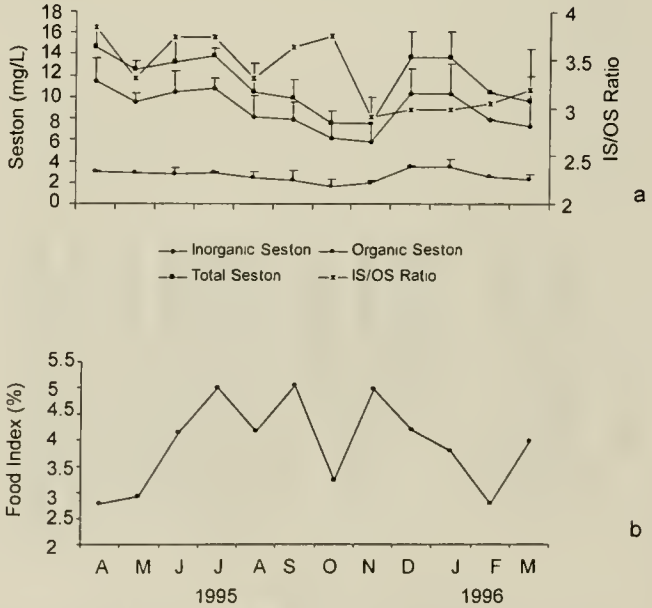

Figure 3. Changes in the seston and IS/OS ratio (a), and food index (b) throughout the annual cycle from the Rada del Puerto del Pichilingue, B.C.S., Mexico. (Error bars = SD).

testinal epithelium to the gonad and therefore to the developing oocytes. In this work, we found a lot of acid mucopolysaccharides in the intestinal loop, the mantle, and the perigonadal connective tissue of the developing gonads (male and female) of *A. ventricosus*. In the gonads, these carbohydrates can be the result of the transference from the intestinal loop (Le Pennec and Beninger 1991) or from the mantle (Barber and Blake 1983). In contrast, in the ripe gonad the acid mucopolysaccharides were few, so we believe that they were used in the maturation of the gametes.

In the developing and ripe oocytes, we observed a lot of glycogen that probably was the result of the transformation of the acid mucopolysaccharides. This carbohydrate is converted into triglycerides and is stored in the oocytes to be used as a future energy source for the larvae (Gabbott 1975). To support this, we found much unsaturated lipids (oil droplets) in the cytoplasm of developing and ripe oocytes.

The gross analysis of the seston or the measurement of a single chemical variable cannot describe fully the nutritive value of seston. To understand seston as food, it is necessary to determine its major biochemical constituents (lipid, protein, and carbohydrate) (Navarro et al. 1993). These components form the food material available for scallops and their larvae. TS, IS/OS ratio, and FI showed no clear relation to the reproductive cycle. It seems the reproductive cycle was influenced by a combination of the quantity of food and the muscle reserves.

A. ventricosus exists in large stocks in the bays of Baja California Sur (Tripp 1985, Aurioles-Gamboa 1992) but some of these stocks have been overfished (Chávez-Villalba and Cáceres-Martínez 1992, Cáceres-Martínez et al. 1993). This is true in Bahía de La Paz, in which the study area of this work is included. There is no fishery in this bay now because of the depletion of the *A. ventricosus* population. A management option is the culture of the species and this idea directed this study. From our results, we can say that the Rada del Puerto de Pichilingue is not an appropriate zone for the culture of catarina scallops. This is because the quality and quantity of food is poor and cannot support commercial production. Though reproductive activity was observed throughout the year as in other locations of Baja California Sur (Baqueiro et al. 1981, Villalejo-Fuerte and Ochoa-Báez 1993, Félix-Pico et al. 1995), the GSI values in the Rada del Puerto de Pichilingue were lower (2% less) than those obtained for *A. ventricosus* from Bahía Concepción, B.C.S., México (Villalejo-Fuerte and Ochoa-Báez 1993).

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BIOECONOMIC ANALYSIS OF A SEA SCALLOP, *PLACOPECTEN MAGELLANICUS*, AQUACULTURE PRODUCTION SYSTEM IN NEWFOUNDLAND, CANADA

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ABSTRACT We report the results of 2-year pilot scale scallop, *Placopecten magellanicus*, culture trials at Charles Arm, in Notre Dame Bay on the northeast coast of Newfoundland during 1989–1991. We used extrapolations of the growth and survival data from these trials, as well as records of all capital, labor, and operational costs, to conduct simulation modeling of cash flows associated with start-up and operation of scallop farms scaled up to commercial size. Our aim was to determine the economic viability of sea scallop farming in Atlantic Canada using the standard economic evaluation methods of Net Present Value (NPV) and Internal Rate of Return (IRR), based on production of 55–65 mm (shell height) live, whole, scallops using a suspended pearl net culture system. Two separate pilot trials were carried out concurrently. In the first, the effect of stocking density and culling on growth and survival were determined by stocking scallop seed in pearl nets at five different starting densities; 50, 75, 100, 150, and 200/net with subsequent culling (thinning) at two different times during grow-out. In the second trial, the effect of seed grading and net mesh size on growth and survival were determined by grading seed into two nominal sizes based on shell height, small (< 18 mm) and large (> 18 mm), followed by stocking in pearl nets of varying mesh size: 4.5, 6.0, and 9.0 mm, at a starting density of 50/net for grow-out. After 2 years of grow-out, size at age (growth) was significantly related to initial seed stocking density, culling, net mesh size, and seed grading. Survival was significantly related to initial stocking density, culling, and seed grading but not to net mesh size. The model simulations predict scallop farming enterprises with vertically integrated culture farms and processing plant operations, and with annual stocking rates of about 1 million or more seed per year, are economically viable in current market and financial conditions. Sensitivity analyses indicate farm business viability is relatively sensitive to changes in sale price received for harvested product, but relatively insensitive to changes in capital costs, labor, other operational costs, or to mortality. The simulations also predict farm ownership of a processing plant enhances the economic viability of farming enterprises. These favorable economic projections support the contention that continued private and government-assisted investment in expansion of a whole scallop culture industry in Atlantic Canada is warranted.

KEY WORDS: Scallop, *Placopecten magellanicus*, aquaculture, bioeconomics

INTRODUCTION

The sea scallop, *Placopecten magellanicus*, has been the mainstay of the traditional commercial scallop capture fishery in Atlantic Canada for many years. Beginning in the 1960s, considerable effort has been expended to explore the commercial aquaculture potential of the sea scallop (Couturier et al. 1995, Naidu et al. 1987) using technology, equipment, and rearing practices widely used in the extensive Japanese scallop culture industry (Taguchi 1977 Aoyama 1989) for the indigenous Japanese scallop, *Patinopecten yessoensis*. North American markets for scallop products have traditionally been almost exclusively restricted to the white adductor muscle, or "meats," as they are known in the industry. Early efforts to commercialize culture of the giant sea scallop, *Placopecten magellanicus*, in Canada's Atlantic provinces were based on production of meats intended to compete in these existing markets.

Development of the culture industry has been slow. Total annual Canadian production of cultured scallop has averaged < 100 mt since 1993 (Dept. of Fisheries and Oceans Statistics Rept. 1998). This figure includes both sea scallop, *Placopecten magellanicus*, production from Atlantic Canada as well as production of the introduced Japanese scallop, *Patinopecten yessoensis*, in British Columbia on Canada's West Coast. Two factors are considered to be primarily responsible for the slow increase in cultured production of Atlantic sea scallop: (1) industry dependence on annual wild seed collection that has proved to be unreliable with wide interannual fluctuations in seed settlement (Couturier et al. 1995); and (2) high production costs for meats that marginalized economic viability (Frishman et al. 1980, Gilbert 1987, Gilbert and LeBlanc 1991, Wildish et al. 1988).

These economic analyses, which focused on "meat" production as the sole source of farm revenue, cited high meat production costs as the principal obstacle to viability but did not consider the harvesting and sale of alternative products. However, markets are reported outside North America for "meats with roe," whole, live scallops and various "value-added" products. In Japan, a large-scale culture industry producing Japanese scallop, *Patinopecten yessoensis*, for sale in a variety of product forms, including whole, in-shell product has thrived for many years (Ikenoue and Kafuku 1992). Whole, in-shell queen, *Chlamys opercularis*, and king, *Pecten maximus*, scallops are marketed in several European countries (de Franssu 1990, Hardy 1991), but availability typically is restricted to markets near fishing ports, because scallops have a relatively short shelf life in air (De Franssu 1990). In British Columbia, live, in-shell, pink scallops, *Chlamys rubida*, and spiny scallops, *C. hastata*, < 80 mm in shell height have been supplied in small quantities (< 100 mt per annum) to both domestic and U.S. markets for several years (W. Heath, BC Min. of Fisheries, pers. comm.). Since 1996, this has been augmented by cultured scallop,

P. yessoensis, production, which has been sold throughout Canada, the U.S., and Asia. Cultured, in-shell sea scallops are also produced in small quantities (< 30 mt/annum) from farms in Nova Scotia and Newfoundland for sale into domestic Canadian markets.

Beginning in 1992, promotional and market development initiatives carried out by Thimble Bay Farms Ltd., have identified a niche market in Canada and the northeastern United States for a 55–65 mm SH (shell height) sea scallop product, sometimes termed "princess" or "cocktail" scallops by the seafood industry, depending upon its intended retail presentation. These are intended for retail and seafood service industry presentations similar to those in existing large volume North American markets for soft-shelled clam (*Mya arenaria*), steamers, littleneck, and cherrystone clams (*Mercenaria mercenaria*), and oysters (*Crassostrea virginica*) (De Franssu 1990). Sale of live, in-shell sea scallops now account for most of the annual farmed scallop production from the private company, Thimble Bay Farms Ltd in Newfoundland.

However, efforts to increase market volume have been limited by unavailability of product attributable in large part to production bottlenecks caused by unstable seedstock supply. This has limited total cultured scallop production in Newfoundland to 10–19 mt annually since 1994 (Dept. of Fisheries and Oceans Statistics Report, 1998). Recent construction of a new scallop hatchery at Belleoram in Newfoundland with an estimated annual production capacity of 20 million seed (G. Deveau, Nfld. Dept. Fisheries and Aquaculture, pers. comm.) may resolve the immediate seedstock supply problem and allow significant expansion in the industry. This has rekindled industry attention toward production and marketing issues, including whether it is economically advantageous to invest in market development for whole scallop products.

Harvest and sale of small, in-shell scallops, in particular, may have a dramatic effect on scallop farm viability. In the United Kingdom, harvest and sale of small (5–6 cm), in-shell queen scallop, *Chlamys opercularis*, is considered to be financially advantageous for scallop farmers, because it reduces labor costs associated with shucking meats and greatly shortens production time (Hardy 1991). This may also be true for sea scallop culture in Atlantic Canada. Recent consulting studies commissioned by the Provincial Government of Newfoundland and Labrador seem to support this contention (Atlantecon 1992, ARA Consulting Group 1993). Both studies developed financial projections suggesting the economic viability of commercial sea scallop culture might be enhanced, as compared to meat production, by developing markets for alternative products, particularly whole scallops < 70 mm in shell height.

The purpose of this paper, is to determine whether continued private industry and government investment in commercial expansion of this sector is warranted. In this paper, we report the results of pilot-scale sea scallop culture trials conducted at Thimble Bay Farms's leased acreage at Charles Arm in Newfoundland, Canada during 1989–1991. These trials sought to determine the biological, technological, and economic factors associated with producing for market a whole, in-shell scallop product using a Japanese suspended pearl net culture system. During the pilot trials, scallop growth and mortality, as well as labor, capital, and operating costs were recorded during a 2-year production cycle. Data collected during the pilot trials were used as input into a financial model to forecast the economic viability of commercial scale farms and, thus, provide both biological and economic bases for capital investment decision making for the Atlantic Canadian shellfish culture industry.

This paper has three goals: (1) to quantify the effects of stocking density, culling during grow-out, initial spat grading, and net mesh size on scallop growth and survival observed in pilot-scale culture trials and use these values to define the optimum husbandry techniques appropriate for future scaled-up commercial operations; (2) to conduct model simulations forecasting the economic viability of commercial-scale sea scallop culture farms utilizing production methodology similar to that used in the pilot trials to produce a 55–65 mm whole scallop; and (3) to assess the impact of vertical integration (e.g., farm ownership of a processing plant along with the culture farm) on projections of economic viability for scallop farming enterprises.

METHODS

Culture Trials

Pilot-scale culture trials were conducted at Charles Arm, in Notre Dame Bay on the northeast coast of Newfoundland. This site is one of two shellfish production areas leased by Thimble Bay Farms Limited, a private shellfish aquaculture company specializing in sea scallop and blue mussels. In October, 1989, 50,000 approximately 1-year-old sea scallop seed, originating from stocks in Port au Port Bay in western Newfoundland were purchased and transferred to the Charles Arm site. Scallop seed were stocked into standard 34-cm square Japanese pearl nets. The pearl nets were hung using a longline, suspended culture system in vertical arrays of 10 nets (Fig. 1). Each vertical array was repeated at 0.7-m intervals along a horizontal subsurface headline suspended at 3-m water depth and supported by surface floats.

Two separate trials were carried out concurrently. In the first, scallop seed were stocked at five different starting densities; 50, 75, 100, 150, and 200/pearl net. All nets were 6-mm mesh size. During the first year of culture in May, 1990, and again, in September, 1990, some of these nets were selected for culling (thinning), while others were left unculled. The nets originally stocked at 50/net were culled to 25/net, while all others were culled to 50/net. In the second trial, seed were graded into two sizes based on shell height, nominally referred to as small (< 18 mm) and large (> 18 mm). The graded seed were stocked in pearl nets of varying mesh size; 4.5, 6.0, and 9.0 mm, at a starting density of 50/net, with the exception of the small size grade which, because of their small size, could not be stocked into the 9-mm nets, because they readily fell through the mesh. All experimental trials were repli-

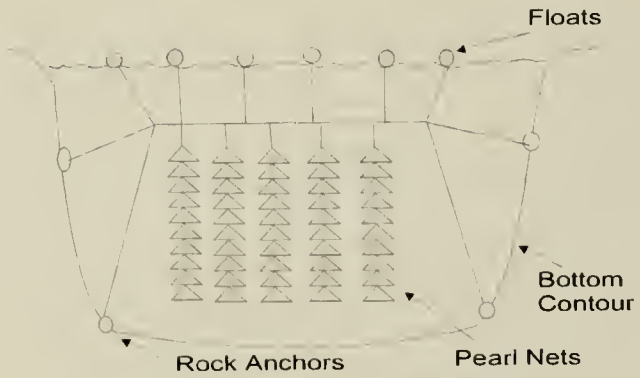


Figure 1. Diagrammatic cross-sectional representation of the longline scallop culture system used in the pilot trials at Charles Arm, Newfoundland.

cated such that each trial had a minimum of six pearl nets (sometimes as many as 10) in each category. In May and September of both 1990 and 1991 (September, 1990 and 1991 only for the size grade-mesh size trial), all pearl nets were retrieved and cleaned by a pressure washer, all scallops were measured for shell height, counted, and all mortalities were removed.

SAS statistical software (SAS Institute, Inc. 1985) was used for all statistical analyses of the biological data from the pilot trials. We used a nested, main effects analysis of covariance model (ANCOVA, SAS Institute Inc., 1985) to determine the relationships among stocking density, culling, size at age (shell height), and survival on each sampling date. Initial starting size of seedstock was the covariate to control for initial variation in shell height among pearl net groups.

Economic Model Parameter Selection

During the culture trials and including the post-trial harvest in September, 1991, records were kept of all capital and operational costs, as well as all labor incurred. These records, as well as the pilot trial growth and survival results, were used as input data to a spreadsheet-based Lotus™ financial model (Table 1) and extrapolated to commercial scale to simulate the startup and operational costs of commercial-scale farms and to forecast their economic viability using the standard financial evaluation methods of net present value (NPV) and internal rate of return (IRR) (Lusztig and Schwab 1977). All equipment, supply, and labor costs were sourced from commercial equipment suppliers as of March, 1999 and are quoted in Canadian dollars. Estimates of useful life span of various equipment were based on practical experience of Thimble Bay Farms. The purchase price of scallop seed and the sale price of harvested product are the most recent values quoted for Thimble Bay Farms, Ltd.

Selection of specific husbandry practices used in the models can have a major impact on the outcome of the model simulations. To ensure parameter values selected were as realistic as possible, we used the results from the stocking density-culling and net mesh-seed grade trials to select appropriate model input values for several key parameters. These included net mesh size, time to harvest for each seedstock cohort, the annual production cycle, stocking density, survival rate to harvest, and frequency of culling and handling for net cleaning.

Although a larger mesh size is expected to yield a faster growth rate, particularly in the second year of the production cycle, the 6-mm mesh is the largest mesh size capable of accommodating the smallest of the purchased seedstock (10–15 mm) in year 1. Any economic advantage attributable to slightly faster growth in 9-mm mesh nets, as compared to the 6-mm mesh, is outweighed by cost considerations because of the need to stock nets of two or more mesh sizes, the utility of which will vary annually, depending on interannual variations in shell height of the seedstock supply. Therefore, use of the 6-mm mesh size was assumed in the simulation modeling exercise.

The minimum time to harvest for each seedstock cohort was set at 15 months. Because the harvesting schedule must be year round, the annual production cycle from each annual seedstock cohort was set at January (year 2) to January (year 3) or, in other words, a 15–27 month production cycle. This production schedule was determined by analysis of the variability in the size at age data from the pilot trials.

Assuming no seasonality in the harvesting schedule, we selected the following stocking scenario for use in the model simulations: 50% of seedstock would be set at 25 scallops/net; 25% at 50/net; 25% at 75/net. Based on the growth data from the pilot trials, this stocking scenario should ensure year-round availability of a 55–65 mm product for harvesting and minimize the likelihood of scallops exceeding the maximum product size specification before being harvested. Although the pilot trials had no pearl nets initially stocked at 25/net, we consider the data from the 50/25 stock culled in May 1990 to represent a reasonable estimate of the probable growth performance of scallops initially stocked at 25/net for use in the model simulations. However, because scallops stocked at 25/net would exceed the maximum acceptable market size in less than 27 months, to ensure year-round availability of 55–65 mm product some seed scallops must be stocked at higher densities. Analysis of the variability in the size at age data from the pilot trials indicated unculled pearl nets initially stocked at 50 or 75 scallops/net best matched the required market size during the 15–27 month production cycle.

TABLE 1.

Selection of key model parameter values used in the economic model simulations.

Key Model Parameters	
Pearl net (square) specifications:	
Cage/mesh size	34 cm/6 mm
Stocking density (% of stock @ # per net)	50% @ 25
	25% @ 50
	25% @ 75
Net clean (# of times per year)	1
Culling/thinning of stock	None
Cost/life span (years) of capital equipment:	
Pearl nets (bulk order)	$1.80/10
Mainline, 365-m coil, 16-mm polypropylene	$98.60/8
Anchor and float lines, 365-m coil, 19-mm polypropylene	$233.10/8
Pearl net droplines, 365-m coil, 7-mm polypropylene	$29.00/8
Floats, 34 cm	$10.50
Floats, 200 L	$35.00
Work boat, 6.8-m aluminum	$9,400/15
Outboard motor, 40 hp	$3,695/5
Boat eqmt., star wheel and hydraulics	$4,000/5
Vehicle, ½ ton pickup with cap	$23,800/5
Processing plant/work shed, 9.3 × 6.2 m	$19,300/20
Plant water pumps	$2,000/5
Hourly labor rate	$8.50
Owner/manager's annual salary	$18,000
Per unit fuel cost (liter, gasoline)	$0.60
Unit cost of autumn-delivered Spat (10–25 mm)	$0.04
Survival rate to harvest	85%
Time to reach harvest size	15–27 months
Harvest schedule	Year Round
Product specifications:	
Market required product size (shell height)	55–65 mm
Ex-plant, per unit scallop sale price	$0.25
Business and startup fees (Year 1)	$9,205–11,505[a]
Crop insurance (per million stock)	$4,000
NPV discount rate (prime + 2%)	8.75%

[a] Varies with farm size.

Individual equipment costs were obtained from commercial supply sources. All other values were obtained from analysis of the pilot trial data or from Thimble Bay Farms Ltd. records.

In the pilot trials, all experimental groups with stocking of 50/net or less achieved survival rates > 85%. Most were > 90%. Therefore, we considered a survival rate of 85% to be a reasonably conservative estimate of survival for the model simulations.

Ideally, operational costs are minimized by selection of husbandry practices that allow individual scallops to be handled as little as possible during the production cycle. Because there are indications from the pattern of survival data, as well as from other concurrent farm operations, that excessive handling has a negative impact on survival, we chose no culling as the preferred production method for the simulations. This also lowered labor costs. For the model simulations, it also necessitated optimizing production solely by varying initial seed stocking density rather than by a combination of stocking density and culling.

The discount rate for NPV calculations was the small business cost of borrowing, as of March, 1999, used by the Canadian banking industry and is calculated as bank prime rate + 2%. A 50:50 split between bank loans and owner equity for capital infrastructure and equipment as well as an operating line of bank credit with a monthly repayment schedule of 3% of the outstanding balance is assumed.

Economic Model Simulations

We selected three hypothetical commercial farm sizes, based on annual seed stocking rates, for the model simulations: 500,000 (½ M), 1 million (1 M), and 3 million (3 M) seedstock per year. The half million size model represents a farm size consistent with a part-time or family operation worked as an income supplement; whereas, the other two represent possible full-time commercial-scale farms consistent with the amount of leased acreage currently utilized by shellfish farms in Newfoundland. Model simulations assume a year-round market requirement for 55–65 mm SH. live, whole product that is fully processed in accordance with all applicable Canadian seafood processing regulations in a farm-owned federally registered processing plant.

The spreadsheet-based financial model (Lotus™) forecasted the potential economic viability of each of these three model farm sizes using the NPV and IRR values. We used a sensitivity analysis procedure to simulate the effect of variability in specific model input parameters on the model output. For the sensitivity analyses, we used an iterative procedure, changing the value of the most important input variables (as a proportion of cash outflow) individually by a pre-set percentage until the NPV at year 10, NPV10 = 0.

To assess the effect of vertical integration (e.g., culture farm plus a farm-owned processing plant) on over-all economic viability of scallop farming enterprises, we recalculated the model simulations with the capital and operational costs of the processing plant deleted. This farm model requires assumption of sale of unprocessed scallops to an ex-farm seafood processor. We used an iterative process, adjusting the ex-farm price for harvested scallops in $0.005 intervals to determine the ex-farm price for unprocessed scallops needed to: (1) achieve minimal standards of economic viability (e.g., $0 < NPV10 < \$1000$; $8.75\% < IRR10 < 9\%$); and (2) achieve economic viability projections for farms without the processing plant comparable to those for the same size farm with the processing plant included.

RESULTS

Density and Culling Trials

At the outset of the culture trial, the mean shell height of all groups ranged from 19–21 mm (Fig. 2). Beginning with the first sampling in May, 1990, shell height was significantly related to stocking density ($P < 0.0001$). This relationship was maintained through all sampling periods. The pattern in least-square means (LSM) among the five initial stocking densities was also significant ($P < 0.01$ or greater) and consistent across all stocking densities ($LSM_{50} > LSM_{75} > LSM_{100} > LSM_{150} > LSM_{200}$).

Shell height was also significantly related to culling ($P < 0.0001$). The LSMs of culled (thinned) scallop groups were consistently larger in shell height than their unculled counterparts ($P < 0.0001$) at the same initial stocking density ($LSM_{cull\text{-}may90} > LSM_{cull\text{-}sep90} > LSM_{unculled}$). The interaction term of stocking density × culling date was also significant ($P < 0.0001$) throughout the sampling period. By the end of the second year of the trial (September 1991) an increase in shell height attributable to culling (Table 2) was noted at most initial stocking densities. In Fig. 2, the slope of the lines between adjacent sampling times indicate the mean growth rate during that interval. Growth rates were highest during the May to September period, 1990 (first summer season) and declined considerably thereafter. The highest mean growth rates, observed in the May 1990 cull group, ranged from 0.142 to 0.176 mm day^{-1}, depending upon stocking density, during this time.

The effects of initial stocking density and culling on survival were less consistent (Fig. 3). Survival was significantly related to both initial stocking density and culling date ($P < 0.0001$). However, the pattern in LSMs was inconsistent among initial stocking densities ($LSM_{50} = LSM_{100} = LSM_{150} > LSM_{75} > LSM_{200}$). LSM patterns with respect to culling date was also inconsistent ($LSM_{cull\text{-}may90} > LSM_{cull\text{-}sep90} = LSM_{unculled}$) although the May 1990 cull group were consistently larger than the other two groups. All except the unculled 200/net group had mean survival rates > 80% at the end of the pilot trials. Most exceeded 85%. Overall, the change in survival attributable to culling was much less pronounced than that for shell height among the experimental groups (Table 2).

Maximum growth was achieved in the 50/25 cull groups. In these groups, more than 90% of all scallops were greater than the minimum acceptable market size by May of Year 2 in the production cycle. Back-calculation of the size at age data from May in Year 2 (1991) based on the mean monthly growth rate during the September, 1990 to May, 1991 period projected that 90% of the 50/25 stock culled in May 1990 were probably in excess of the 55 mm minimum market size in January of Year 2 (1991). Thus, the minimum time to first harvest is approximately 15 months.

Seed Grading and Mesh Size Trials

We used a similar analytical approach to determine the relationships among seed grading and net mesh size with size at age (shell height) and survival. When graded, the mean shell height of scallop seedstock in the two nominal size grade categories were 15.3 mm (small grade) and 22.5 mm (large grade). At the end of Year 2 of the pilot trial in September, 1991, size at age was significantly related to both mesh size ($P < 0.0001$) and initial size grade ($P < 0.03$). The interaction term was not significant ($P > 0.05$). All groups exceeded 45 mm shell height by the end of Year 1 and exceeded 60 mm shell height by the end of Year 2 (Fig. 4a). Increasing mesh size had a positive effect on mean size at age for both size grades. However, the mean shell heights of small size grade groups were sometimes larger at the end of Year 2 compared to large size grade seed in nets of the same mesh size ($LSM_{9L} = LSM_{6S} > LSM_{6L} > LSM_{4.5S} = LSM_{4.5L}$).

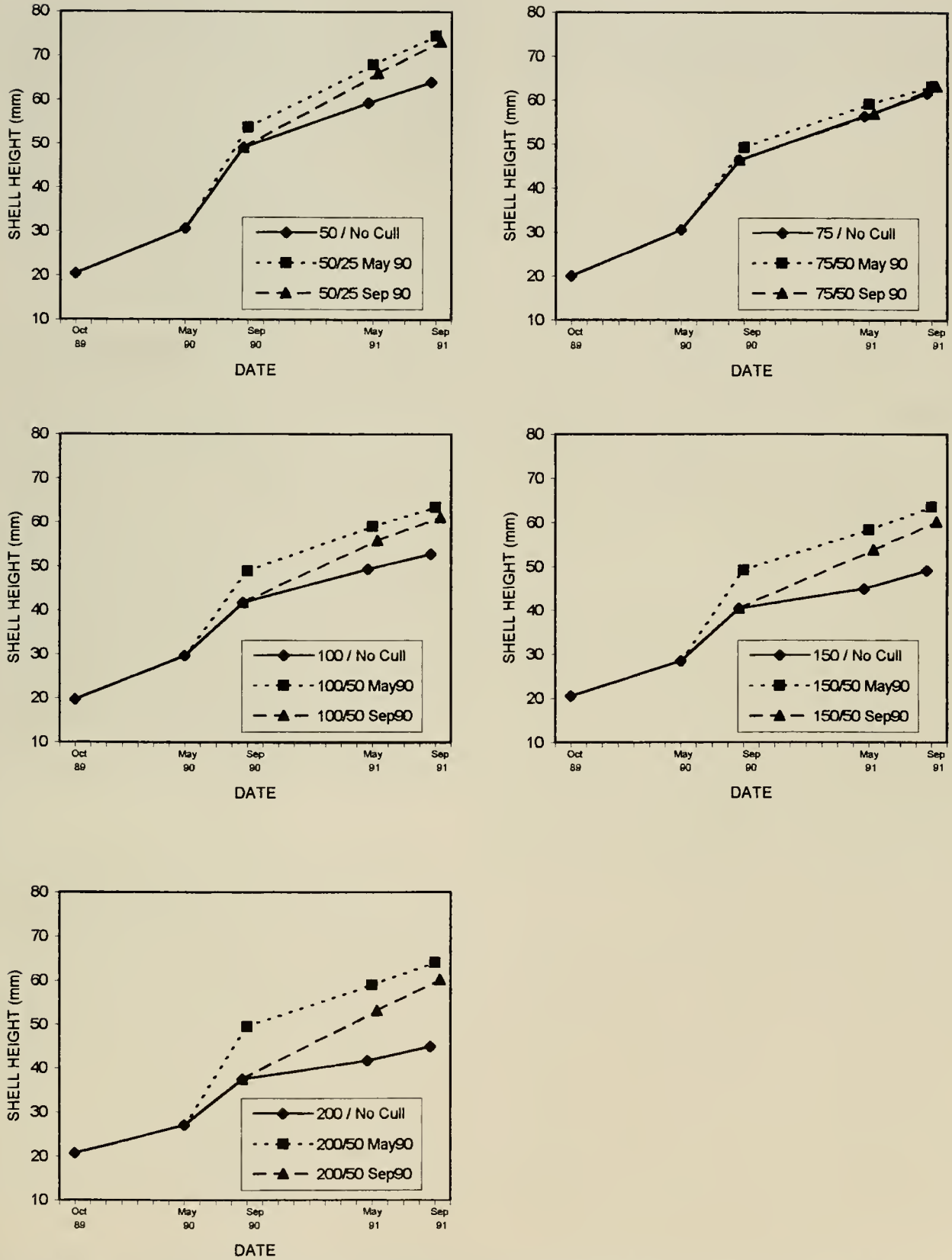


Figure 2. Mean size at age (shell height) over time of scallops in the stocking density-culling trials. The figure legends follow the convention "original stocking density / culled density, and date of culling" (e.g., 50/25 May90 means original stocking density = 50, culled density = 25, and May 1990 was the date of culling). Standard error bars (± 2 SE) are plotted but are obscured by the datapoints.

TABLE 2.

Percentage (%) change in mean shell height and mean survival at Year 2 attributable to culling in Spring (May) and Autumn (September) of Year 1, compared to the unculled stock of the same initial stocking density. Stocking density values are number of scallops net^{-1}.

	Shell Height		Survival	
Stocking Density	May Cull	Sept. Cull	May Cull	Sept. Cull
50	16.2	14.2	0.5	−2.7
75	2.5	2.6	12.0	5.3
100	20.2	15.7	−3.7	−6.7
150	29.4	22.7	0.1	4.4
200	42.5	33.9	24.4	5.7

Survival through Year 2 was not significantly related to net mesh size ($P > 0.05$) but was weakly related to initial size grade ($P < 0.04$). The small size grade experienced a relatively lower survival during the trials (Figure 4b) than did the large grade ($LSM_{9L} = LSM_{4.5L} = LSM_{6L} > LSM_{6S} = LSM_{4.5S}$). Survival through Year 2 in all groups exceeded 85%, while mean survival of the large grade exceeded 91%. In September, 1991, the observed mean size at age and mean survival of scallops in the mesh size-seed grade trials were comparable to those observed in the stocking density-culling trials.

Effect of Farm Size

The key parameter values used as input to the model simulations are given in Table 1. Net cashflow projections for all three farm sizes followed similar patterns of an initial cash investment in business startup (year 0), a further negative net cash outflow in the first year of operation, followed by a series of positive net cash inflows in subsequent years, the magnitude of which increased with increasing farm size (Fig. 5). Net present value (NPV) and internal rate of return (IRR) values derived from the model simulations indicate both the 1 M and 3 M farms are projected to be economically viable using a 10-year forecast horizon at present commercial bank interest rates and market prices (Figure 6). The smaller (½ M) farm size is not considered economically viable. The model simulations predict a trend of increasing NPV and IRR values with increasing farm size indicating the influence of "economy of scale" in farm operations.

Annual labor and seedstock acquisition costs represent > 50% of the total cash outflow for all farm sizes (Fig. 6). Acquisition of capital equipment and infrastructure is a relatively smaller proportion of cash outflow when annualized over the 10-year model simulation cycle. However, much of the cost for capital equipment and farm infrastructure are concentrated in Year 1 (processing plant, work boat, culture equipment, etc.). Labor and debt servicing costs as proportions of total cash outflow over a 10-year cycle do not vary with farm size. However, acquisition of capital equipment and operational costs both decline proportionally with increasing farm size; whereas, purchase of annual seedstock proportionally increases over a 10- year period. The payback period, defined as the time to recoup the initial investment assuming operating profits are retained within the business, is estimated at 4.2 and 3.4 years for the 1 M and 3 M farms, respectively.

Sensitivity Analyses

To determine how robust our viability projections from the model simulations were, we recalculated the simulations for the two model farm sizes deemed economically viable with the base input assumptions (1 M and 3 M farms). We used an iterative procedure, changing the value of each of the most important input variables (as a proportion of cash outflow) individually by a preset percentage until the NPV10 = 0. Projections of economic viability for both the 1 M and 3 M farm models are relatively insensitive to changing value assumptions for most major input variables, including capital, operational costs, and mortality (Fig 7). However, both models are relatively sensitive to changes in sale price. Reduction in sale price obtained for harvested product in the order of 20% and 28%, for the 1 and 3 M farms, respectively, reduced the NPV to zero. This is equivalent to a minimum sale price of $0.20 and $0.18/scallop, respectively.

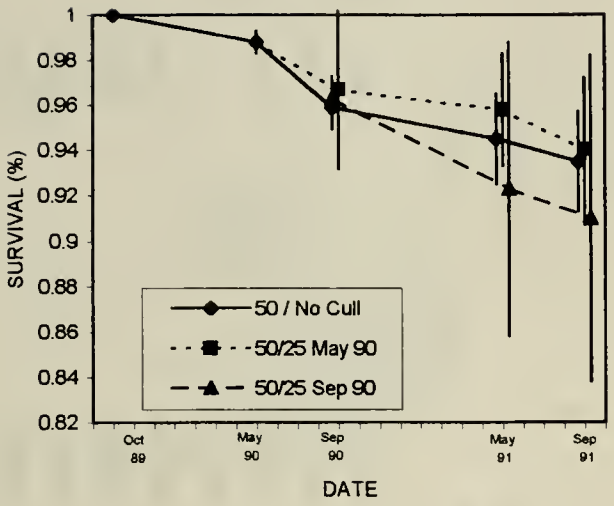
Effect of Farm-Owned Processing Capacity

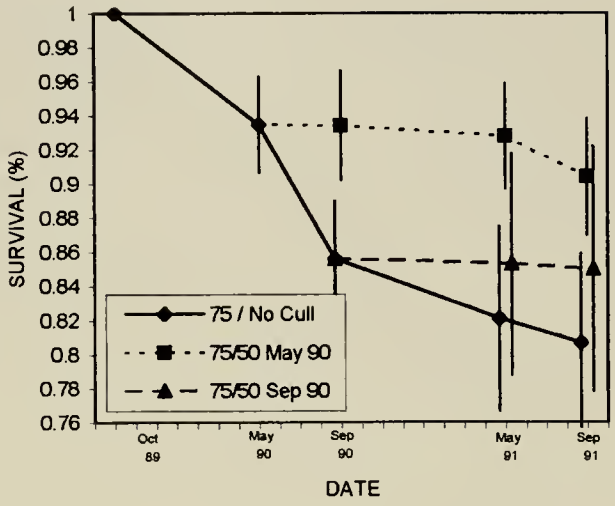
Without the processing plant, the ½ M farm is still not considered to be viable economically (NPV10 < 0; IRR10 < 8.75%) under assumptions of current scallop sale prices. Farms of this size only become marginally economically viable (e.g., 0 < NPV10 < $1000; 8.75% < IRR10 < 9%) if the ex-farm sale price for unprocessed scallops exceeds $0.26/scallop, a price that exceeds the current sale price for processed scallops. For the 1 M and 3 M farms without processing plants, economic viability becomes marginal as the ex-farm sale price for unprocessed scallops approach $0.185 and $0.165/scallop, respectively. To achieve economic viability projections comparable to those for farms with processing plants (equivalent NPV or IRR), the sale price for ex-farm unprocessed scallops must exceed $0.235/scallop for both the 1 M and 3 M farms, a difference of only $0.015/scallop for unprocessed versus processed scallops at current prices. Obtaining such a small price differential (approximately 6%) for sale of unprocessed scallops to an ex-farm processor may not be realistic, because it would seem to allow a rather small profit margin for the processor. With this considered, scallop farming enterprises with owner-operated processing capacity are likely more economically attractive than farms without owner-operated plants.

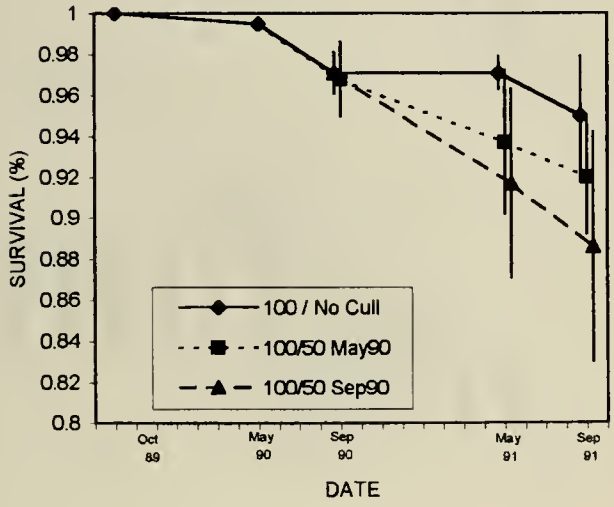
DISCUSSION

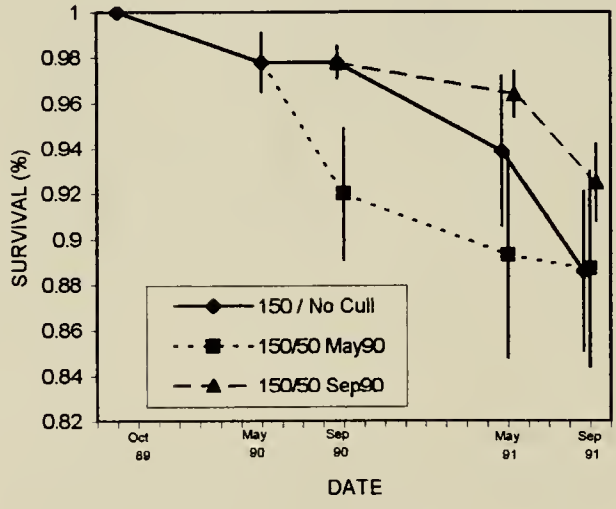
In commercial production systems, growth and survival are the two major biological rates of importance to cultured seafood growers. For bivalve mollusks, many factors influence these two variables. Some are environmental, such as food availability and water temperature, and others are physiological related to age, size, and reproductive maturity of the animals themselves (see Shumway 1991 for review). For suspended culture systems, additional stock husbandry factors must also be included, such as gear depth, type of gear and mesh size, current velocity, stocking density, and extent of biofouling (Claereboudt et al. 1994a, Claerboudt et al. 1994 b, Côté et al. 1993, Parsons and Dadswell 1992, Parsons and Dadswell 1994, Shellfresh Farms Ltd. 1993).

In this paper, we have examined the effect on scallop growth and survival of the major variables that can be readily manipulated by scallop farmers, assuming use of a basic pearl net culture system styled after the equivalent Japanese industry for the Japanese scallop, *Patinopecten yessoensis*. These are selection of stocking density, gear mesh size, culling (thinning) practices, and seed grading. In their review of sea scallop culture in Atlantic Canada, Couturier et al. (1995) considered stocking density the single most important factor affecting cultured sea scallop growth rates. Although it is difficult to compare growth rates, size at age, or sur-









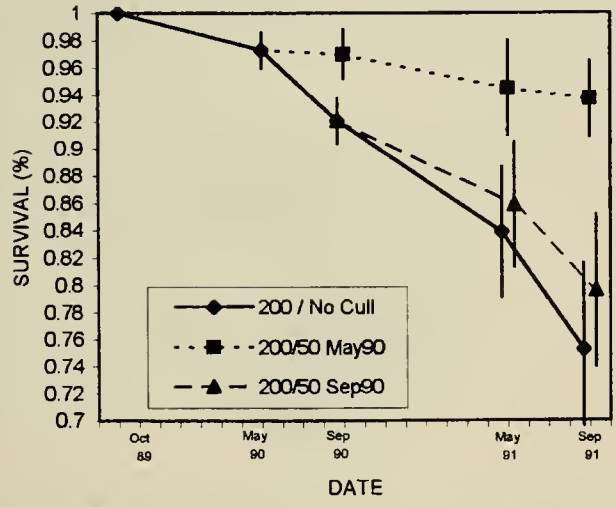


Figure 3. Mean survival over time of scallops in the stocking density-culling trials. The figure legends follow the convention "original stocking density / culled density and date of culling" (e.g., 50/25 May90 means original stocking density = 50, culled density = 25, and May 1990 was the date of culling). Standard error bars are ± 2 SE.

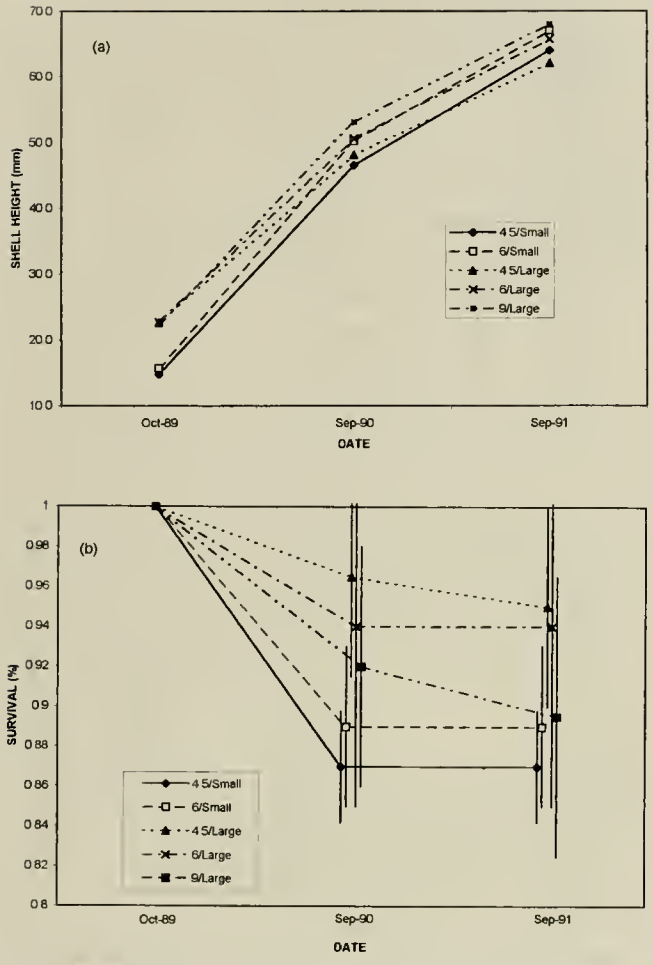


Figure 4. Mean size at age (shell height) and mean survival of scallops in the spat grading-net mesh size trials. The figure legends follow the convention "net mesh size in millimeters / nominal spat size grade at time of stocking" (e.g., 4.5 / small means mesh size 4.5 mm and stocked spat were small grade). Nominal spat size grades were < 18 mm (small) and > 18 mm (large). Standard error bars are ± 2 SE but are obscured by the datapoints in (a).

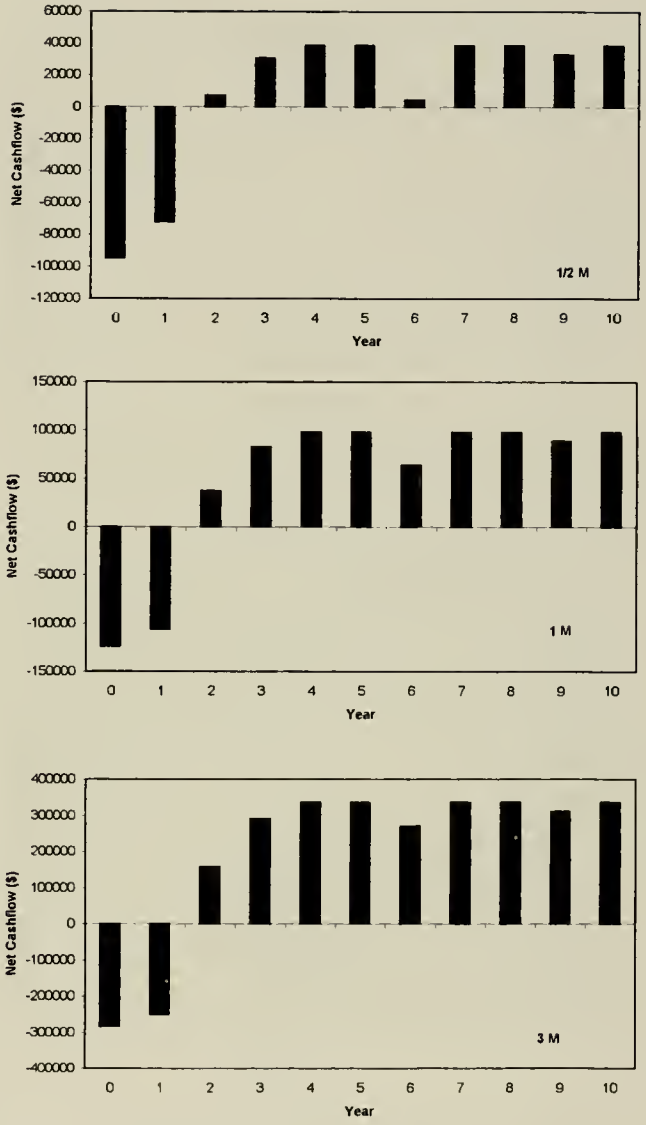


Figure 5. Annual net forecasted future cashflows over a 10-year period from initial startup for three sizes of sea scallop farms with farm-owned processing plant included: ½ million seed yr^{-1}, 1 million seed yr^{-1}, and 3 million seed yr^{-1}. Year 0 is the initial capital investment before startup.

vival data among different studies, gear, and locations, the growth and survival rates observed in this study seem consistent with those reported for sea scallops in suspension culture grown elsewhere in Atlantic Canada (Côté et al. 1993, Dadswell and Parsons 1991, Parsons and Dadswell 1992, Parsons and Dadswell 1994, Wildish et al. 1988).

In our study, size at age and survival varied with stocking density, gear mesh size, culling practices, and seed grading. Both size at age and survival tended to decrease with increasing stocking density, a result consistent with previous studies on several scallop species including the Japanese scallop, *Patinopecten yessoensis* (Yamamoto 1978, Ventilla 1982), the bay scallop, *Argopecten irradians* (Duggan 1973, Rhodes and Widman 1984), and the sea scallop, *Placopecten magellanicus* (Côté et al. 1993). However, Penney (1995), in a study of large scallops > 75mm shell height, did not find a significant relationship between stocking density and survival. Parsons and Dadswell (1992) also found survival in sea scallops from New Brunswick to be unrelated to stocking density. In the present work, nonrandom handling mortality, an artifact of our "batch processing" style sampling procedure, was likely implicated in the inconsistent survival patterns among the different density groups observed in the pilot trials. This might explain the anomalous high mortality among the 75/net groups. Similar instances of nonrandom apparent handling mortality among adjacent groups were noted periodically during the farm's other commercial operations. We, therefore, consider excessive or improper handling of gear to be a more important factor influencing survival than stocking density, a finding that would be consistent with that of other studies (Parsons and Dadswell 1992, Ventilla 1982, Wildish et al. 1988).

In this work, size at age in pearl net culture was improved by early culling and by increasing the initial net mesh size. Survival was also improved by early culling but was unrelated to net mesh size. Larger sized seed did not maintain their size at age advantage over small grade seed after 2 years of grow-out. This suggests shell height variation in 1-year-old seed scallops from wild sources is

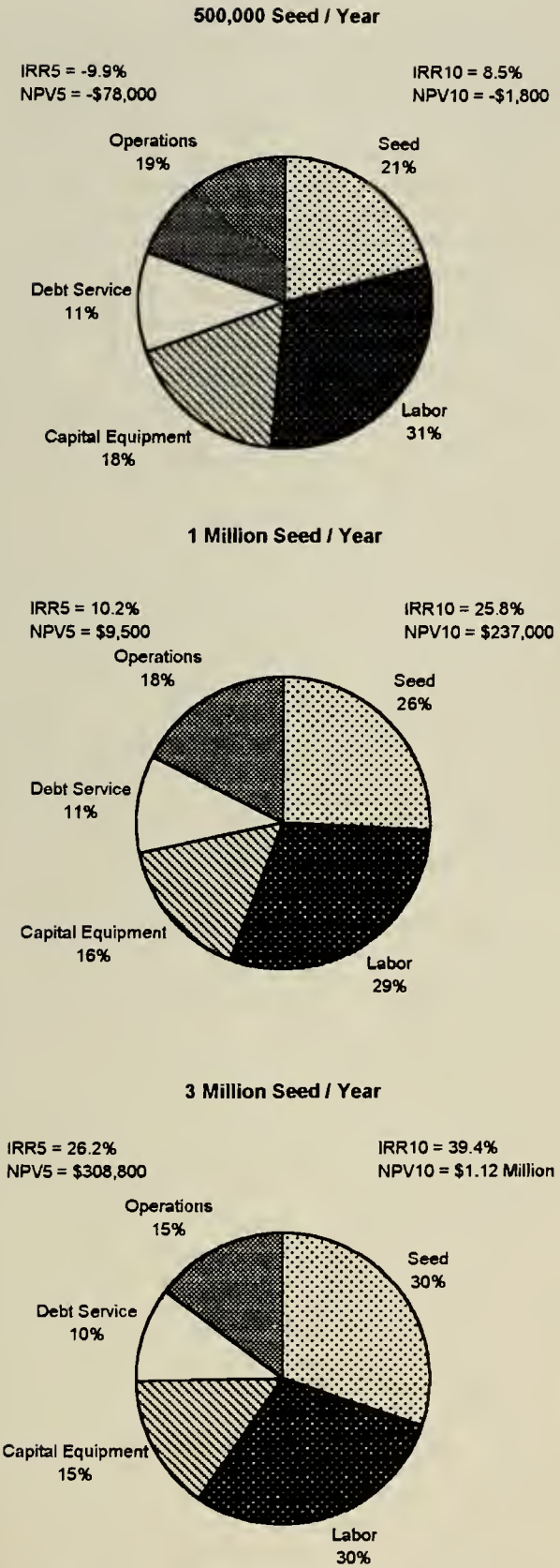


Figure 6. Mean annual cash outflows by category over a 10-year cycle, with NPV and IRR values for model simulations of three sizes of farm operations. (NPV5 = NPV calculated over 5 years, etc.). NPV and IRR calculations were based on cashflows from Figure 5.

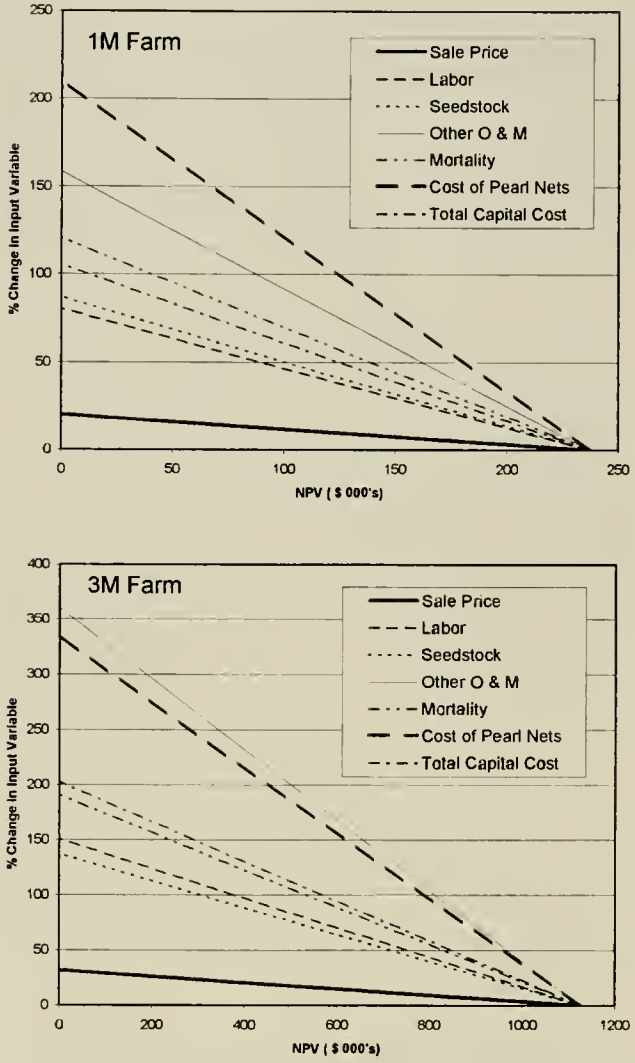


Figure 7. Sensitivity analysis of the effect of changing value assumptions of the major model input variables (reduction in sale price; increase for all cost variables and mortality) on projected NPV10 values (NPV calculated over 10 years) for the 1 M and 3 M farm models. Percentage change in input variables at NPV = 0 indicate the proportional change from the base values for each variable required to reduce NPV10 to zero.

likely the result of variation in environmental factors or seed collection husbandry practices rather than within population genetic variation. However, seed grading before initial stocking may have commercial value as a means to reduce size at age variation within individual pearl nets at harvest time.

Selection of production practices for the model simulations was guided by two general considerations: (1) the intended product was a 55 to 5 mm, whole, live scallop; and (b) harvesting and sale of product was required year-round with no seasonality in production volume. Ensuring year-round availability of a 55 to 65 mm product requires adjustment of farm production practices, including selection of stocking densities, deciding whether or not to cull and when, and selecting a harvesting schedule to minimize labor costs, while still meeting market demands. Variable stocking densities are required to ensure correctly sized stock are available for harvest throughout the year. Economic viability is optimized by early generation of sales revenue, achieved through timely harvesting.

However, harvesting should not be so early that within-net shell height variation is such that a significant proportion of scallops in harvested nets are under the minimum acceptable market size. This situation would necessitate the return of large numbers of undersized scallops to pearl nets for further on-growing, resulting in added labor cost.

It would seem unlikely that further minor changes to the basic pearl net culture system or husbandry practices are capable of affecting significant improvement in labor costs and, hence, the outcome of the model simulations. The sensitivity analyses indicate the viability of model farms is relatively insensitive to changes in capital costs, labor, or other operational costs. Further significant reductions in labor costs are likely to be achieved only by substitution to another culture gear entirely or by use of increased mechanization during farm operations. Parsons and Dadswell (1994) suggested that, although the initial capital cost of pearl nets was much lower compared to lantern nets, when handling times and their associated labor costs were factored in, lantern nets and Shibetsu nets gave the lowest over-all cost of production. However, this suggestion must be viewed with some caution. In a previously reported scallop rearing trial in Newfoundland using scallops > 75 mm shell height, scallops raised in pearl nets were larger than those raised in lantern nets at comparable stocking densities (Penney 1995). In the same study, survival was unaffected by gear type.

The reason for the better growth in pearl nets is unknown, but it may be attributable to differences in water flow (and, hence, food availability) around and within the two net types. All suspended net culture systems impede water flow, which, in turn, negatively affects production, a condition that is exacerbated by increased stocking density, reduced net mesh size, and biofouling (Claereboudt et al. 1994b, Devaraj and Parsons, 1997, Parsons and Dadswell 1994). Pearl nets are estimated to reduce water flow by 46–61% (Claereboudt et al. 1994b), but no comparative measurements are available for lantern nets. The better growth of scallops in pearl nets compared to lantern nets (Penney 1995) support selection of the basic pearl net system as the more appropriate net type compared to lantern nets. In addition, some growers find large lantern nets clumsy to handle from small boats similar to those used in our simulations.

Projections of economic viability derived from the model simulations indicate commercial sea scallop farms marketing a whole, 55–65 mm product can be profitable enterprises in Atlantic Canada. Our favorable projections are in sharp contrast to earlier economic analyses for culture systems based on production of adductor meats alone for sale into traditional North American scallop markets (Frishman et al. 1980, Gilbert 1987, Wildish et al. 1988, Gilbert and LeBlanc 1991).

These conflicting economic projections for in-shell versus meat production are likely because of a combination of factors. Revenues from in-shell product sales begin about 15 months after stocking in our model simulations. In contrast, production of meats in the 30–40 count range (North American scallop markets quote in number of pieces to make one pound weight) would require an extra 15 to 20 months of culture (Penney 1995; Penney and McKenzie, 1996) and would likely generate less ex-farm revenue per scallop at current North American market prices (Urner Barry 1999). This protracted culture time would also increase capital costs, because extra pearl nets and other gear are required for each annual seed cohort, as well as increase labor costs for stock thinning, gear deployment, and in-plant meat shucking.

In Newfoundland, only two companies are currently in commercial production although this will likely increase quickly, because a total of nine companies and 11 culture sites in various stages of development totaling nearly 400 leased hectares are now in operation (G. Deveau, Nfld. Department of Fisheries and Aquaculture, pers. comm.). Recent annual scallop production by the two farms currently selling cultured scallops has varied since 1994 from 10–19 mt, the majority of it marketed in whole form. Estimated annual production from a single 1 M farm would be about 17 mt. This is approximately 10–20% of all in-shell scallop products currently being sold in Canada from Canadian sources. Clearly, considerable developmental marketing initiatives would be required by industry to expand significantly North American market share for in-shell scallop products sufficient to absorb the production of a new Atlantic industry composed of several such farms. Alternatively, the potential for increased development of other international export markets into such countries as France, with an existing tradition of acceptance of alternative scallop products (de Franssu 1990) should be determined.

A trend of increasing NPV and IRR values with increasing farm size indicates significant economies of scale accrue to larger farms. Despite farm size, annual labor and seedstock acquisition costs are the largest factors in over-all annual cash outflows. The cost of labor has been previously recognized as an important component of over-all production costs for scallop culture (Atlantecon 1992, Parsons and Dadswell 1994). In our model simulations, labor is reduced through elimination of the need for culling during grow-out by selecting appropriate initial stocking densities. This tends to improve the over-all survival rate as well. Larger sized farms (1 M and 3 M models) are projected to be more profitable than smaller operations (½ M model).

In Atlantic Canada, many shellfish culture farms, particularly in mussel and oyster culture, have been started as "cottage-style" ventures operated on a part-time basis as a source of supplementary family income by persons employed in other industries. If started by families already employed in the fishing industry, economic viability forecasts using NPV or IRR calculations typically remove from consideration certain capital costs (e.g., cost of boat, motor, truck, ropes, etc.) that are shared with the fishing enterprise (e.g., Ridler 1995). These capital costs are considered to have been already compensated by the fishing enterprise. The ½ M model is sufficiently small in scale to be considered this way. If calculated using these assumptions, the ½ M model is forecasted to be economically viable (NPV = $34,000; IRR = 16.5%).

Of particular interest for industry development purposes, is the effect the owner-operated processing plant has on projected economic viability. Vertical integration and increased farm size are known to have a positive effect of the viability of other shellfish aquaculture operations (Adams and Pomeroy 1992, Lambregts et al. 1993). For scallop farming enterprises, irrespective of farm size, NPV- and IRR-based projections of economic viability changed only slightly with elimination of the owner-operated processing plant as part of the over-all enterprise. This is attributable to two factors. First, the capital investment in processing capacity is quite small for processing whole scallops (see Table 1). Because product processing of whole scallops consists of a fairly simple process of washing and cleaning shells, sorting empty shells, and packaging, a relatively small building with minimal equipment is needed. Second, the labor costs for such a simple processing operation are also relatively minor. Processing in-shell scallops

eliminates the need for shucking, which is the most labor-intensive component of in-plant scallop "meat" processing.

For either the 1 M or 3 M farms, comparable NPV and IRR values were projected for enterprises with and without processing capacity at a sale price difference of only $0.015 per scallop for ex-farm processed scallops versus ex-farm unprocessed scallops. It seems unlikely that ex-farm processing companies would pay to the farmer such a small price differential for unprocessed scallops, because this leaves them a very tight margin for their own capital and operational processing costs and potential profit. It is far more likely that processing companies would pay farmers a lower price for unprocessed scallops, which would have a negative impact on the economic viability projections for farming enterprises. Thus, incorporation of an owner-operated processing plant as part of the business venture would be likely to enhance business viability. Ex-farm prices for unprocessed scallops in the range of $0.16–$0.18/scallop would make scallop farming not economically viable under current conditions regardless of farm size. Nevertheless, product processing with subsequent direct sale of product to seafood buyers and brokers represents a level of business management and marketing activity that some prospective farmers may not choose to pursue. This may be especially true for the ½ M farm size model that may be a part-time or family operation.

Favorable NPV and IRR projections are not the only criteria upon which to evaluate the potential for success of any new business venture. It should be recognized that many other factors can and do influence individual business investment decisions that are not considered in NPV or IRR calculations. Other factors, such as timing of large cash outflows versus revenues, debt repayment schedules, other financing arrangements, personal, biological, legal, and regulatory considerations, all may vary on an individual business and location basis and may also affect the success of any business venture (Lusztig and Schwab 1977).

Both the NPV and IRR financial forecasting methods used in our analyses are based on the estimation of future cash flows generated by an initial capital investment and are commonly used as decision-making tools by financial analysts to guide investment in new businesses. Both give explicit consideration of the time value of money, incorporated through the discounting of cash flows, which is often related back to the cost of credit (borrowing) from banks. Accurate forecasting of future cash flows, the basis of successful NPV and IRR applications, is often a challenge (Lusztig and Schwab 1977) and cannot anticipate aperiodic potentially catastrophic events, such as disease outbreak, major loss of gear because of ice damage, etc. Despite these individual situational considerations, favorable general NPV and IRR values such as we have forecaste from our model simulations indicate the underlying potential profitability of sea scallop farming in Atlantic Canada. Our positive NPV and IRR projections for in-shell scallop farms indicate continued industry and/or government investment to encourage commercial expansion in this sector is warranted.

Sensitivity analyses indicate the forecasted profitability is fairly robust with respect to anticipated variability in capital and operating costs, and stock mortality, but is relatively sensitive to fluctuations in sale price for harvested product. This last point must be closely considered in the start-up of any commercial business venture of the scale outlined by the model simulations. Greatly increased product availability in the marketplace may exert downward pressure on prices, particularly in the presence of inadequate marketing efforts. Given the in-shell nature of the product, prices may not be affected by trends in market prices for traditional scallop meats, a critical point considering the continuing increase in Chilean and Chinese cultured meat production as well as past fluctuations in both price and supply of meats from the North American fishery (de Franssu 1990). In existing North American markets, an in-shell sea scallop product would be more likely to compete (and, hence, to be affected by price fluctuations) with soft-shelled clam (*Mya arenaria*), steamers, littleneck, and cherrystone clams (*Mercenaria mercenaria*), and oysters (*Crassostrea virginica*).

We consider further development of a sea scallop farming industry in Atlantic Canada to be constrained by four factors: (1) availability of a reliable large-volume annual seedstock supply at commercially acceptable prices; (2) market development necessary to substantially increase the current, small volume niche-market status of North American markets for whole, 55–65 mm products; (3) the reported short shelf life of live scallops (de Franssu 1990) and; (4) the long-term frequency and severity of shellfish site closures because of outbreaks of biological toxins. The first two are inextricably linked. Resolution of the seed supply problem that has plagued industry expansion for years (Couturier et al. 1995), possibly by increased hatchery production of seedstock, will greatly increase the volume and interannual stability of available harvested product and, thus, encourage greater market penetration of the 55–65 mm whole product. Large-scale markets for whole scallop products will only be developed when production volume is sufficient to warrant the required financial investment for promotional market development. However, live scallops are reported to have a relatively short ex-farm shelf life as compared to other molluscan shellfish such as clams, oysters, or mussels (de Franssu 1990). Increasing market volume for whole scallop products may require a shift from sale of live product to a frozen in-shell or other secondarily processed form. This would have a negative impact on our projections of farm enterprise economic viability unless accompanied by commensurate farm-gate price increases.

Ultimately, the limitation to increased production of whole scallop products in Atlantic Canada most difficult to mitigate may be that caused by the distribution, frequency, and prevalence of biological toxin outbreaks. Scallop species are well known for their propensity to sequester biological toxins in their mantle, roe, and hepatopancreas tissue at relatively high levels as compared to other bivalve species (Shumway and Cembella 1993; Douglas et al. 1997). Detoxification of affected scallops may be quite slow, exceeding several months in duration and be quite variable among individuals (Shumway and Cembella 1993). Frequent and severe toxin outbreaks may limit expansion of scallop farms to areas where toxin outbreaks are relatively infrequent and of short duration. Although this has been the case in Newfoundland, thus far, continued industry expansion, particularly elsewhere in the Atlantic Canadian provinces may be seriously impeded by toxic event considerations.

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THE EFFECT OF CYTOCHALASIN B DOSAGE ON THE SURVIVAL AND PLOIDY OF *CRASSOSTREA VIRGINICA* (GMELIN) LARVAE

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ABSTRACT Survival and ploidy of D-stage oyster larvae (*Crassostrea virginica*) were determined following the rearing of embryos exposed to CB dosages of 0.5 mg/L, 0.25 mg/L, and 0.125 mg/L for 10 minutes, with 0.05% DMSO and ambient seawater as controls. The experiment was replicated three times on the same day with the same procedures and partially stripping the same male oysters; different females were used for each replicate. CB dosage treatments began when 50% of the eggs reached PB1 (24–31 min). Embryos were reared for 48 h at ambient temperature and salinity. Resulting triploid percentages were 13% ± 6.7% (0.125 mgCB/L), 61.8% ± 6.2% (0.25 mgCB/L), and 68.2% ± 14.1% (0.5 mgCB/L). No significant difference ($P \leq 0.05$) in mean survival was found between the three CB treatments. Significant differences in mean survival between the three replicates implies variability because of different sources of eggs.

KEY WORDS: *Crassostrea virginica*, oyster, triploid, cytochalasin B, dosage

INTRODUCTION

Cytochalasin B (CB), a cytokinetic inhibitor, was first used to produce triploid *Crassostrea virginica* and *Crassostrea gigas* (Thunberg) over a decade ago (Allen 1986, Stanley et al. 1981). Optimal treatments; that is, those yielding high proportions of triploids, have been reported for *C. gigas*, based on temperature, dosage, time of application, and duration; namely, 0.5 mgCB/1mL dimethyl sulfoxide (DMSO)/L of seawater for 20 min at 25 °C, when 50% of the eggs were at meiosis I (Allen et al. 1989, Downing and Allen 1987). Because *C. virginica* is less fecund than *C. gigas* (Galtsoff 1964), there is more concern for egg survival. Lower dosages and treatment times of 0.5 mgCB/L for 15 min at 25 °C (Shatkin and Allen 1990) and 0.25 mgCB/L for 10 to 15 min, at 27 to 29 °C (Barber et al. 1992) were suggested to increase the survival of embryos while maintaining high yields of triploids.

We tested the feasibility of triploid *C. virginica* production in Louisiana, based on the premise that higher summertime meat yields resulting from triploidy could be profitable for the oyster industry. Triploid induction, using 0.5 mg/L CB, was variable with commercial size broods (≥ 4 million eyed larvae). During the first summer of commercial-scale production, survival of CB-treated embryos was ≤ 5% compared to ≤ 21% for diploid controls using stripped gametes. Differences between the salinity at our hatchery and salinities at sites where broodstock were obtained affected development time, in particular meiotic synchrony, and have been identified as major causes of this variation (Supan 1995).

The objective of this study was to investigate the effect of CB dosage (H_0:$\mu_{0.5mgCB} = \mu_{0.25mgCB} = \mu_{0.125mgCB} = \mu_{0mgCB}$; H_a:≠) on survival and triploidy induction in *C. virginica*, and to determine what component of the variability was attributable to females, held in identical environments.

METHODS AND MATERIALS

Survival and ploidy of oyster larvae were estimated after exposing embryos to CB dosages of 0.5 mg/L, 0.25 mg/L, and 0.125 mg/L for 10 min, with 0.05% DMSO and ambient seawater as controls. The experiment was replicated three times on the same day with the same procedures by partially stripping the same male oysters; different females were used.

Preparation of Gametes

Gametes were obtained for each replicate in a fashion similar to the method described by Allen and Bushek (1992). Oysters were collected from nearshore containers, opened, and their gender was determined microscopically using gonadal smears. Ripeness was visually recognized by the presence of prominent genital canals. Female and male oysters were placed in separate areas to avoid cross contamination.

Eggs were obtained from three ripe females, randomly chosen for each replicate. Females were individually dry-stripped (i.e., without using seawater) to ensure equivalent periods of hydration (defined as the length of time eggs are exposed to seawater after stripping) and simultaneous fertilization. The resulting eggs were pooled and washed of gonadal debris with filtered (1 μm) ambient (24‰) seawater (FAS) by passing them through a 75 μm Nytex screen onto a 15 μm screen. They were then resuspended for enumeration and brought to a volume of approximately 8 million in 1 L FAS. The eggs were allowed to hydrate for 60 min at 28 °C before fertilization and treatment.

Three male oysters were partially stripped for each replicate by scraping away only a portion of the gonad into a beaker and then covering the oyster with plastic wrap to prevent desiccation. Sperm from the three males was pooled in a beaker after being washed of gonadal debris by passage through a 15 μm screen.

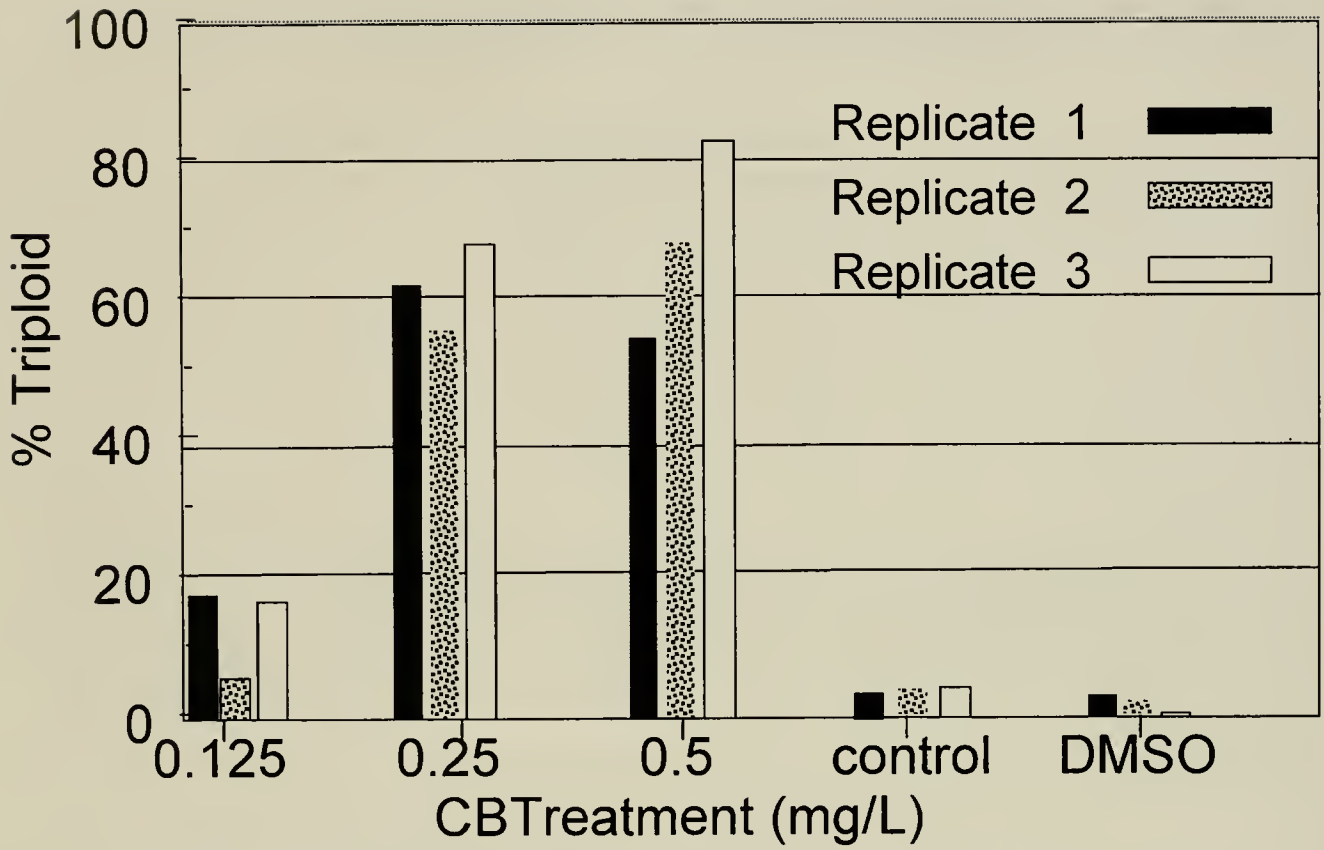

Figure 1. Percentage triploidy in *C. virginica* D-stage oyster larvae after treatment with cytochalasin B, by replicate.

Fertilization and Treatment

Pooled eggs were fertilized with approximately 10 sperm/egg and stirred regularly. After fertilization of the 8 million eggs, they were divided into five treatment beakers each containing 800 mL of FAS, bringing the eggs per treatment to about 1.5 M eggs/L. Eggs from individual beakers were examined microscopically for polar body formation at appropriate intervals. Treatments began when approximately 50% of the eggs reached PBI (24 to 31 min among replicates).

Treatments consisted of adding the appropriate aliquot of 1 mg CB/1 ml DMSO to the beakers of developing eggs to obtain dosages of 0.5 mg/L, 0.25 mg/L, and 0.125 mg/L. Our control consisted of 0.05% DMSO (*v/v*) dissolved in FAS and FAS alone served as a normal. Treatments lasted for 10 min. Afterward, each CB-treatment group of embryos was rinsed of CB with FAS over a 15 μm screen then placed in separate beakers containing 0.05% DMSO-FAS solution for 15 min. The embryos from each beaker were then rinsed of the DMSO solution and put into separate, labeled culture vessels containing 15 L of FAS for a final culture density of 15 embryos/L. Culture vessels were aerated and equal volumes *Isochrysis* aff. *galbana* clone CISO added. Embryos were incubated for 48 h at ambient temperature and salinity until they reached D-stage. All counts were obtained using triplicate 1 mL subsamples from each culture vessel. At 48 h, each vessel was individually drained onto a 40 μm screen, and subsamples were placed into 1.5 mL centrifuge tubes and shipped overnight to Rutgers University's Haskin Shellfish Research Laboratory for ploidy determination using flow cytometry.

TABLE 1.

Results of analysis of variance (ANOVA): Effect of cytochalasin B treatment and experimental replication on the percentage triploidy of *C. virginica* oyster larvae.

Sources of Variation	DF	F-ratio	Prob > F
Treatment	4	49.70	0.0001
Replicate (error)	2	0.69	0.5291

$R^2 = 0.9616$.

TABLE 2.

Results of ANOVA: *Post hoc* comparisons of mean percentage triploidy of *C. virginica* larvae by treatment.

	Triploidy*		
Treatment	Mean	SD	Comparisons**
0.125 mg CB	0.3594	0.110	A
0.25 mg CB	0.9052	0.063	B
0.5 mg CB	0.9783	0.156	B
Control w/o DMSO	0.1862	0.009	A
Control w/DMSO	0.0949	0.084	A

* Triploidy = arcsin ($\sqrt{(\%\text{Triploid})}$ (0.01)).
** Tukey's honestly significant difference ($\alpha = 0.05$).
SD = Standard deviation.

Data Analyses

Differences among treatment means for survival and percentage triploidy were determined using analysis of variance (SAS 1991). Percentage triploidy was determined as a proportion of triploid cells among the total number analyzed by the curve-fitting program ModFit (Verity Software House, Topsham, ME) (Allen and Bushek 1992). Survival and percentage triploidy met the assumptions of normality and variance homogeneity after angular transformation (Dowdy and Wearden 1991). The models used survival and percentage triploidy as separate dependent variables and treatments and experimental replicates as independent variables. Tukey's Honestly Significant Difference Procedure was used to test the difference among the treatments and replicates ($\alpha = 0.05$).

RESULTS

Percentage triploidy and survival were not different between 0.5 mg/L and 0.25 mg/L CB treatments.

Percentage Triploidy

In treatments, mean percent triploidy was 13% ± 6.7% for 0.125 mgCB/L, 61.8% ± 6.2% for 0.25 mgCB/L, and 68.2% ± 14.1% for 0.5 mgCB/L. In controls, 1.4% ± 1.3% of the 0.05% DMSO treatment and 3.4% ± 0.3% of the FAS normal larvae were triploid. Figure 1 depicts percentage triploidy by treatment and replicate. Variation seems high among the three replicates; however, transformed data revealed no significant difference ($P < 0.05$).

The model (% triploidy = treatments, replicates) defined the relationship between the treatment effects and percentage triploidy and explained most of the variability ($R^2 = 0.9616$). Treatment was highly significant ($P < 0.0001$), and the replicate effect was not significant ($P = 0.5291$) (Table 1). *Post hoc* comparisons of mean percentage triploidy found neither significant differences between the 0.125 mgCB/L and the two controls, nor between the 0.25 mgCB/L and 0.5 mgCB/L dosages (Table 2).

Survival

Figure 2 shows survival by treatment and replicate. On average, the results demonstrate an inverse relationship between survival and CB dosage and a lack of effect (slight enhancement) with DMSO exposure. Although there was moderate variability among the replicates, overall, they all demonstrated the same trends across treatments.

The model explained a reasonable amount of variation in survival ($R^2 = 0.7172$). Both replicate and treatment were highly significant ($P < 0.0001$, Table 3). For treatments, both control and normal were the same, and all CB groups were the same (Table 4). Overall, CB groups had about 20% lower survival than did the controls.

DISCUSSION

These results support previously reported findings that 0.25 mgCB/L (Barber et al. 1992) and 0.5 mgCB/L (Shatkin and Allen 1990) are appropriate dosages for inducing triploidy in *C. virginica*. However, results are variable depending upon egg or sperm quality or some other factor (Allen and Bushek 1992).

Treatment Recommendations

We found no statistical difference in percentage triploidy or survival between the two higher CB dosages. Considering the cost

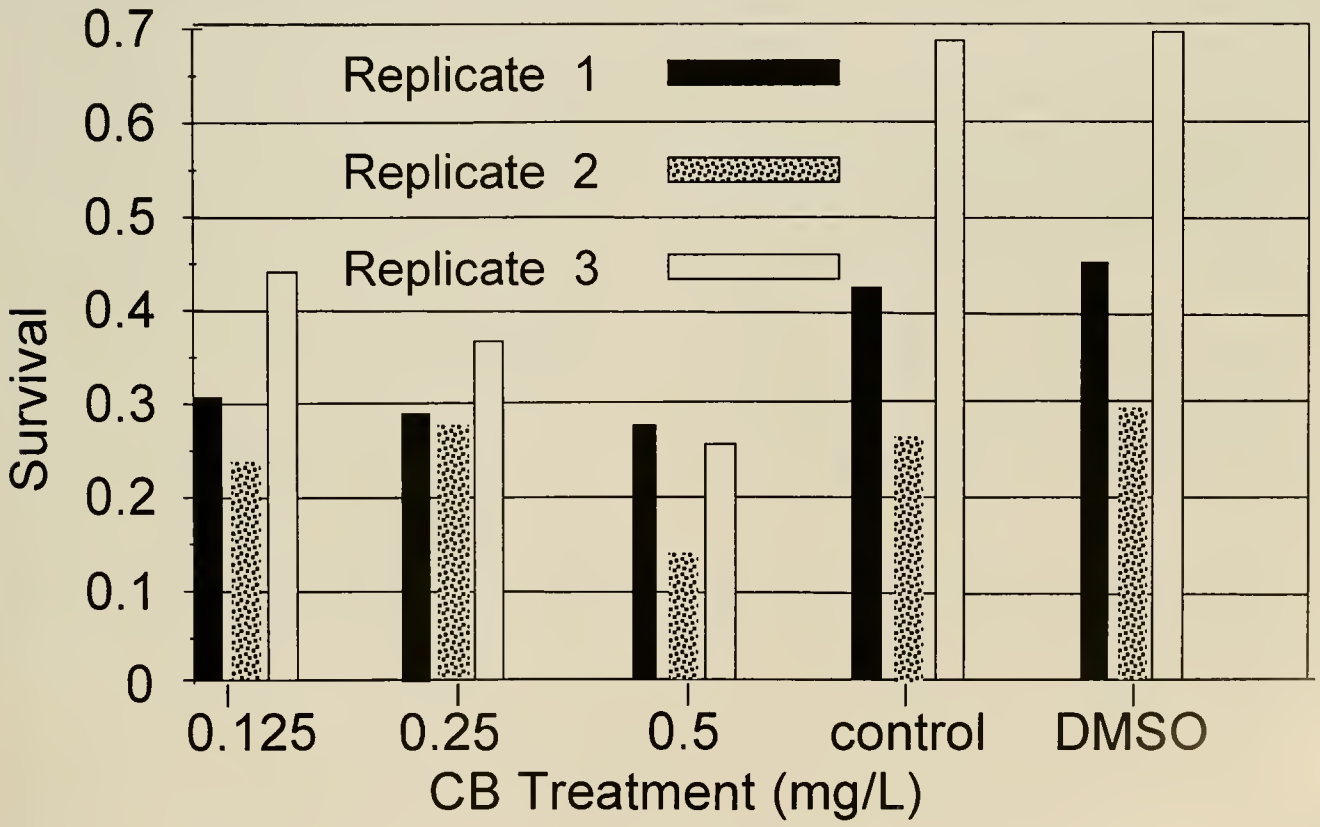

Figure. 2. Survival of *C. virginica* embryos to D-stage larvae after cytochalasin B treatment, by replicate.

TABLE 3.

Results of ANOVA: Effect of cytochalasin B treatment and experimental replication on survival of *C. virginica* larvae.

Sources of Variation	DF	F-ratio	Prob > F
Treatment	4	11.54	0.0001
Replicate (error)	2	25.11	0.0001

$R^2 = 0.7172$.

TABLE 4.

Results of ANOVA: Means and standard deviations (SD) of survival of *C. virginica* by treatment with comparisons.

Treatment	Survival* Mean	SD	Comparisons**
0.125 mg cb	0.5961	0.1204	A
0.25 mg cb	0.5795	0.0860	A
0.5 mb cb	0.4748	0.0913	A
Control w/o dmso	0.7334	0.2035	B
Control w/dmso	0.7585	0.2126	B

* Survival = arcsin ($\sqrt{\text{(Normal larvae/embryos)}}$).
** Tukey's honestly significant difference ($\propto = 0.05$).

of CB ($10/mg in the U.S.), economics suggest that the lower effective dosage is preferable, at 28 °C for 10 min. However, with a range of 54 to 82% triploidy (0.5 mgCB/L) versus 55 to 67% (0.25 mgCB/L), one is inclined to use the higher dosage. Greater triploidy might have resulted from a longer (15 min) treatment time, at the sacrifice of lower survival. For maximum triploid production, embryos should be exposed to CB for a period of time long enough to have a high proportion captured at PB1 extrusion but short enough to minimize mortality (Barber et al. 1992). Allen and Bushek (1992) attributed low variance in triploid production to using meiosis I as a benchmark to begin treatment, claiming to have effectively removed meiotic rate as a factor. Although the time of initiation is determined by an appropriate developmental milestone (i.e., 50% PB1), the duration is fixed and does not accommodate varying meiotic rates. We suggest that appropriate duration of treatment be addressed by using a developmental benchmark to cease treatment as it is used for beginning it. Observation of a subsample of eggs, held at the same temperature but without treatment, could provide such a cue. Although this cue must be determined empirically, we suggest 2–5% cleavage might be appropriate. This benchmark could be used for *C. virginica* or any bivalve species.

The real solution to improving efficiency of triploid production is the development and use of tetraploid broodstock. Tetraploid male oysters produce diploid sperm; when used to fertilize eggs from diploid females, 100% triploid offspring result (Guo and Allen 1994).

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DELIVERY OF RIBOFLAVIN TO LARVAL AND ADULT PACIFIC OYSTERS, *CRASSOSTREA GIGAS* THUNBERG BY LIPID SPRAY BEADS

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ABSTRACT Lipid spray beads (SB) were prepared containing 13% w/w particulate riboflavin. Beads suspended in seawater lost 73% riboflavin after 24 h. Release of riboflavin from SB ingested by Pacific oyster (*Crassostrea gigas*) larvae was observed under epifluorescent light. Riboflavin concentrations in tissues of adult oysters fed on riboflavin-SB were significantly (SNK; $P < 0.05$) greater than those of oysters fed on seawater-filled SB. Concentrations of riboflavin in oysters exposed to dissolved riboflavin were not significantly greater than those of oysters fed on seawater-filled SB, indicating that elevated riboflavin concentrations in oysters fed on riboflavin-SB were attributable to breakdown of ingested beads rather than uptake of dissolved riboflavin leaked from SB into the culture medium. SB seem to be a promising means of delivering water-soluble nutrients to bivalve suspension feeders

KEY WORDS: Spray beads, lipid, riboflavin, oyster, larvae, *Crassostrea gigas*

INTRODUCTION

Little is known of the nutritional requirements of bivalve mollusks despite their obvious importance in aquaculture and natural ecosystems. The main reason for this lack of knowledge is that nutritionally satisfactory, defined artificial diets are not available. The development of microparticulate diets that both retain dietary ingredients when suspended in seawater and are digestible by bivalve mollusks has been a difficult goal to achieve. High surface area to volume ratios of microparticulate diets together with low molecular weights of essential nutrients, such as water-soluble vitamins, results in their rapid loss. For example, López-Alvarado et al. (1994) reported that > 80% amino acids were lost from microgel particles after only 2 minutes in aqueous suspension.

To address the problem of rapid loss of water-soluble nutrients from microparticulate feeds, Langdon and Siegfried (1984) developed lipid-walled microcapsules for the delivery of water-soluble vitamins to juvenile oysters (*Crassostrea virginica*). Later Buchal and Langdon (1998) and Langdon and Buchal (1998) developed lipid spray beads (SB) for the delivery of water-soluble nutrients and therapeutic substances to bivalves. Buchal and Langdon (1998) found that it was important to soften the walls of lipid-walled capsules and SB by adding 40% w/w fish oil to the tripalmitin walls of the particles in order to make them digestible by clams (*Tapes philippinarum*); however, softening the walls of SB in this way lowered 24-h retention efficiencies for encapsulated riboflavin from 97.9 to 85.1% (Buchal and Langdon 1998).

Seguineau et al. (1996) reported that the microalgal species *Isochrysis galbana*, *Pavlova lutheri*, and *Skeletonema costatum* contained high concentrations of riboflavin and thiamine; however, the concentrations of these two vitamins in scallop (*Pecten maximus*) larvae fed on a mixture of these algal species declined during growth and development. Seguineau et al. (1996) suggested that microencapsulated supplements of riboflavin and thiamine could be used to study the requirements of scallop larvae for these vitamins.

In this paper, we describe the results of feeding experiments in which larval and adult oysters (*Crassostrea gigas* Thunberg) were fed on SB containing particulate riboflavin to evaluate the potential usefulness of SB in delivering low-molecular weight, water-soluble nutrients to bivalve mollusks.

METHODOLOGY

Spray Beads

Preparation of Spray Beads Containing Riboflavin

Spray beads were prepared containing micronized, particulate riboflavin (Sigma) based on the method described by Buchal and Langdon (1998). Briefly, riboflavin crystals were ground to a fine powder (< 5-μm particles; McCrone micronizing mill, McCrone Scientific Ltd). Two grams of ground powder were mixed by sonication at 90 °C with 8 g of a lipid mixture made up with 4.8 g tripalmitin (Fluka Chemical Co.) and 3.2 g of menhaden oil (light cold pressed; Zapata Haynie Ltd.). The heated mixture was then forced under pressure (90 psi) through at atomizing nozzel (SUE-25B; Spraying Systems Ltd.) supplied with pressurized nitrogen at 10 psi. The beads were collected in a stainless steel cylinder cooled with liquid nitrogen then stored in the dark at −20 °C until use.

Determination of Encapsulation Efficiency

To determine encapsulation efficiencies, 10 g of SB were first dissolved in 3 mL chloroform and the vitamin core material extracted by addition of 3 mL distilled water with shaking. The aqueous supernatant was removed and the extraction repeated three times. Aqueous extractions were combined and the concentration of dissolved riboflavin determined spectrophotometrically (absorbance at 267 nm).

A subsample of 0.5 mL of chloroform was removed from the capsule extraction and transferred to a dry, tarred weighing boat. The chloroform was removed by heating for 24 h at 50 °C, and the boat was reweighed to determine the weight of extracted lipid.

Encapsulation efficiencies were expressed as the weight of encapsulated vitamin (mg) per 100 mg of lipid.

Retention of Riboflavin by SB Suspended in Seawater

Retention of riboflavin by SB was determined by measuring the proportion of initially encapsulated riboflavin remaining after 24 h suspension in seawater. To prepare SB for a leakage experiment, beads were first suspended in 2% polyvinyl alcohol with sonication. SB were then sieved using a 40-μm sieve, and beads under 40 μm were collected on a GF/C filter and rinsed with cold (5 °C) distilled water. The beads were then washed from the filter with cold distilled water and collected in a sealed vial and stored at 5 °C in the dark.

At the start of a leakage experiment, 75 to 100 mg of the sieved (< 40 μm) SB were suspended in 15 mL seawater (20 °C) by vigorous shaking. Immediately after suspension (t = 0), 1 mL of the bead suspension was taken and filtered onto a GF/C filter. The filtered SB were then washed with 1 mL of chilled (5 °C) seawater. The filtrate and washings were pooled and stored in the dark at 5 °C for analysis. Riboflavin concentrations were determined as described above. The remainder of the SB suspension was placed on a continuous agitator at 20 to 22 °C. Samples of suspended SB were taken over a period of 24 h to determine changes in the retention of riboflavin over time. Retention efficiency (RE) was defined as

$$\mathrm{RE} = \frac{\text{riboflavin retained on the filter}}{\text{riboflavin on filter and in filtrate}} \times 100$$

Breakdown of SB and Release of Riboflavin by Larvae

Feeding experiments were conducted to determine if oyster larvae could ingest and breakdown SB, thereby releasing riboflavin into the digestive system. Broodstock oysters were spawned, and the resulting larvae were raised on a mixed diet of *Isochrysis galbana* (T-ISO) and *Chaetoceros calcitrans* (Breese and Malouf 1975). After 8 days of culture, larvae were sieved onto a 45-μm screen, divided into two groups and each resuspended in two liters of seawater at a density of 10 larvae per mL.

Riboflavin-SB at a concentration of 50 SB/μL were fed to one group of larvae with gentle aeration to maintain SB in suspension. After 1 hour, larvae were sieved from suspension using a 45-μm sieve, rinsed with seawater, then resuspended in two liters of filtered seawater and fed on T-ISO alone for 2 hours. The larvae were then sieved from the culture medium and preserved with 0.5% formaldehyde (final concentration made up in seawater, buffered at pH 8.0 with borax) for microscopic analysis. The second group of 8-day old larvae were fed on T-ISO alone for 2 hours, then sampled and preserved for microscopic analysis (as described above).

Sampled larvae were examined using an epifluorescent microscope (Leica DMRBE; excitation 355–425 nm, emission 525 nm) at ×400 magnification. Green and yellow fluorescence indicated the presence of dissolved and particulate riboflavin, respectively, while red fluorescence indicated the presence of chlorophyll derived from ingested algae.

Breakdown of SB and Uptake of Released Riboflavin by Adults

An experiment was carried out to determine if adult oysters could breakdown ingested SB and absorb released vitamin into the hemolymph and tissues. Three groups of six adult oysters were separately held in 20 L of seawater and fed for 6 hours on T-ISO at a concentration of 50 cells/μL in combination with one of the following additions:

1. 20 riboflavin-SB/μL (equivalent to a concentration of 1.15 mg riboflavin/L or a vitamin dose of 3.8 mg riboflavin/oyster);
2. 20 seawater-filled SB/μL; or
3. dissolved riboflavin at the same concentration provided in 1.

Two grams of riboflavin-SB were suspended in distilled water, and the suspension was then poured through a 20μm mesh sieve. SB smaller than 20 μm were collected and filtered onto a GF/C filter, rinsed, and resuspended in 10 mL of distilled water. Aliquots of 100 μL SB suspension were taken to determine riboflavin and bead concentrations. Riboflavin concentrations were determined as described above. SB concentrations were determined using a Coulter Counter (Model TA2). Seawater and food additions were replaced every 2 hours over a period of 6 hours. The cultures were gently aerated to maintain beads in suspension.

After 6 hours, oysters were removed and dissected. Hemolymph samples were taken with a syringe from both the heart and the sinus of the adductor muscle. Samples of stomach contents were removed with a Pasteur pipette inserted through the mouth. Tissue samples of mantle and adductor muscle were also removed. The samples were frozen at −20 °C for protein and riboflavin analysis.

Riboflavin and Protein Analysis

Hemolymph samples were centrifuged, and riboflavin concentrations of the supernatant fraction were determined by high-pressure liquid chromatography (HPLC) (Seguineau et al. 1996). Mantle and adductor muscle samples were ground in 0.2M HCl and 0.6N perchloric acid (PCA) and centrifuged. Supernatants were then removed, their volumes adjusted to 2 mL with distilled water and riboflavin concentrations determined by HPLC (Seguineau et al. 1996).

Protein concentrations of hemolymph samples were determined by the method of Bradford (1976), using bovine serum albumen as a standard. Treatment of adductor muscle and mantle samples with HCl and PCA for the extraction of riboflavin probably resulted in the precipitation of most proteins in these samples; therefore, Bradford assays indicated concentrations of PCA-soluble protein and peptides in muscle and mantle samples. Riboflavin concentrations were expressed in terms of ng riboflavin per mg protein in tissue samples or per mL of stomach extract.

Statistics

The rate of loss of riboflavin from SB suspended in seawater was analyzed by regression analysis. Riboflavin concentrations in oyster samples from the three experimental treatments were compared by analysis of variance (ANOVA) (Model III; Super ANOVA, Abacus Concepts). Concentrations were log-transformed to ensure homogeneity of variances, as determined by plots of residual values against fitted values. If ANOVA indicated a significant treatment effect on riboflavin concentration, individual treatments were compared by Student–Newman-Keuls (SNK) multiple range test (significance level $P < 0.05$).

RESULTS

Encapsulation and Retention Efficiencies

SB were found to have an encapsulation efficiency of 13% w/w (mg riboflavin per 100 mg lipid). Leakage experiments indicated

that almost half the encapsulated riboflavin was lost from SB during the first 3 hours of suspension in seawater, followed by a more gradual loss over the subsequent 21 hours (Fig. 1). Approximately 27% of the initially encapsulated riboflavin was retained after 24 hours of suspension, equivalent to 3.5 mg of riboflavin retained per 100 mg of lipid.

Regression analysis indicated that there was a significant (P = 0.0012) relationship between the log of the fraction of riboflavin retained and the duration [log time (h)] that SB were suspended in seawater (Fig. 1). The rate of loss of riboflavin could be expressed in terms of the equation:

Log fraction retained = $-0.116 - [0.333 \times \log \text{time (h)}]$ r^2 = 0.994

Breakdown of SB and Release of Riboflavin by Larvae

Larvae were able to ingest and breakdown SB, liberating encapsulated riboflavin into the digestive system. Free riboflavin was observed as a diffuse greenish fluorescence in the guts of larvae fed on SB, and riboflavin crystals present in intact or partially digested SB were evident as bright yellow points (Fig. 2). The digestive systems of larvae fed on algae alone fluoresced red because of the presence of chlorophyll from ingested algae but no yellow or green fluorescence was evident (Fig. 2).

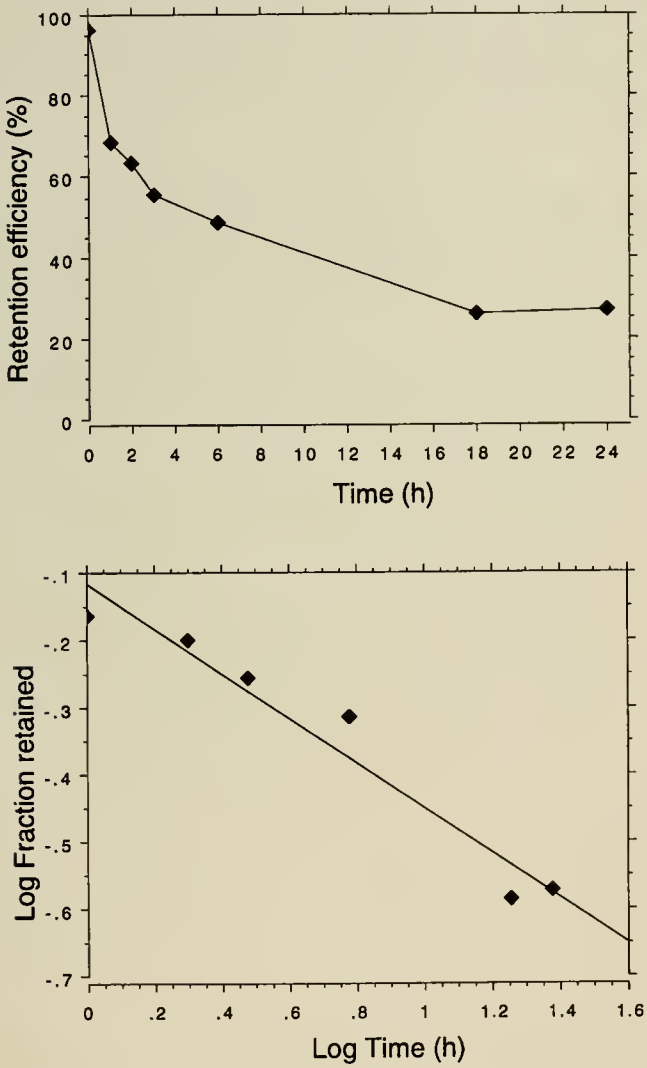

Figure 1. Retention of riboflavin by lipid spray beads suspended in seawater. Top: Change in percentage riboflavin retained by beads over a 24-h period Bottom: Relationship between log of fraction retained and log time duration of beads suspended in seawater. Log fraction retained = $-0.116 - [0.333 \times \log \text{time (h)}]$; $r^2 = 0.994$

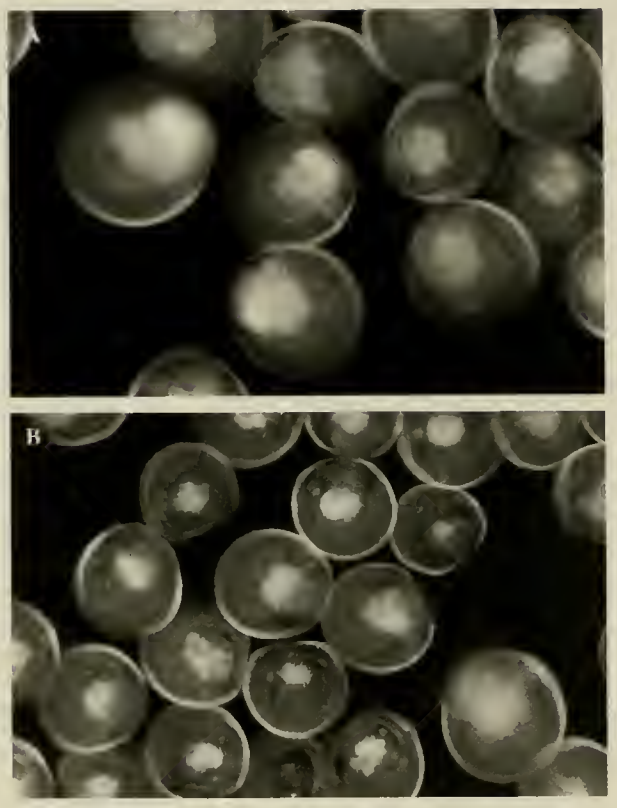

Figure. 2. Eight-day-old larvae of the Pacific oyster (*Crassostrea gigas*) viewed under epifluorescent light (excitation 355–425 nm, emission 525 nm) at ×400 magnification. Average larval shell length = 122 µm. Top: Larvae fed on riboflavin-containing lipid spray beads (50 beads/µL) for 1 hour, followed by a 2- hour period of feeding on T-ISO alone. Bottom: Larvae fed on *Isochrysis* sp. (T-ISO) alone for 2 hours.

Breakdown of SB and Uptake of Released Riboflavin by Adult Oysters

ANOVA of log-transformed riboflavin concentrations in oysters fed on riboflavin-SB were significantly greater (SNK; $P < 0.05$) than those of oysters either fed on seawater-filled SB or exposed to riboflavin dissolved in seawater (Table 1). The presence of significantly higher concentrations of riboflavin in the hemolymph, adductor muscle, and mantle of oysters fed on riboflavin-SB indicated that oysters were able to digest the lipid walls of SB and absorb released riboflavin. There were no significant differences in riboflavin concentrations in hemolymph sampled from either the heart or adductor muscle (ANOVA; $P > 0.05$).

Concentrations of riboflavin in the tissues of oysters exposed to dissolved riboflavin were not significantly (SNK; $P > 0.05$) different from those of oysters fed on seawater-filled SB, indicating a limited ability of adult oysters to take up dissolved riboflavin from seawater.

CONCLUSIONS

Feeding experiments indicated that both oyster larvae and adults were able to breakdown SB and release riboflavin. Free

TABLE 1.

Concentration of riboflavin in tissues of adult Pacific oysters exposed to either lipid spray beads (SB) containing 13% riboflavin at a concentration of 20 SB/μm, seawater-filled SB at a concentration of 20 SB/μL or dissolved riboflavin at the same concentration as that delivered by riboflavin-SB (1.15 mg/L).

Treatment	Stomach Contents (ng/mL)	Hemolymph		Mantle (ng/mg PCA-soluble protein)	Adductor muscle (ng/mg PCA-soluble protein)
		Heart (ng/mg protein)	Muscle (ng/mg protein)		
Seawater-filled SB	20 ± 4	47 ± 19	21 ± 10	198 ± 94	177 ± 115
Dissolved riboflavin	24 ± 4	84 ± 53	51 ± 32	112 ± 22	67 ± 20
Riboflavin-SB	7274 ± 1619	1844 ± 660	1165 ± 247	1633 ± 573	10636 ± 3808

Values are given as means (±1 SE, $n = 6$).

riboflavin was evident in the stomachs of larvae and elevated riboflavin concentrations were evident in the tissues of adult oysters after being fed on riboflavin-SB. Adult oysters exposed to concentrations of dissolved riboflavin, equivalent to those supplied by encapsulated riboflavin, did no show elevated tissue concentrations of riboflavin, indicating that uptake of dissolved riboflavin lost from SB was not a significant source for adult oysters.

About half of the riboflavin was lost during the preparation of SB, based on a comparison between the measured encapsulation efficiency of 13% and a maximum theoretical encapsulation efficiency of 25%. Further losses of riboflavin from SB occurred after suspending SB in seawater; for example, it can be estimated (based on Eq. 1) that 39% of encapsulated riboflavin would have been lost at the end of each 2-hour period of the adult feeding experiment.

In this study, retention of riboflavin by SB suspended in seawater for 24 h was only 27% compared with 85% reported by Buchal and Langdon (1998). This difference may have been attributable to higher encapsulation efficiencies of SB used in this study, because riboflavin-SB prepared by Buchal and Langdon (1998) had an encapsulation efficiency of 4.8% compared with an encapsulation efficiency of 13% for SB used in this study.

The effects of additions of riboflavin-SB on the growth and survival of oysters needs to be determined in future experiments. Because of the need to prepare SB with a high proportion (> 60%) of lipid wall material to ensure encapsulation of the core material, it is unlikely that SB will be useful in delivering bulk dietary ingredients, such as protein and carbohydrate, to oysters. However, SB may be useful in supplementing algal or artificial diets with water-soluble micronutrients, such as essential amino acids or water-soluble vitamins (Seguineau et al. 1996).

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MODELING SEASONAL PROLIFERATION OF THE PARASITE, *PERKINSUS MARINUS (DERMO)* IN FIELD POPULATIONS OF THE OYSTER, *CRASSOSTREA VIRGINICA*

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ABSTRACT A temperature-disease course model was developed to predict the effect of seasonal water temperature changes on disease progression of Dermo in field populations of *Crassostrea virginica*. A linear model was used to describe the relationship between weighted prevalence (disease intensity) and lagged cumulative temperature, where cumulative temperature was used as an estimate of cumulative harm. The model developed for Long Island Sound showed the strongest relationship between cumulative temperature and disease intensity when a lag time of 53 days was used. Point and interval estimates for the day(s) of the year when a mean weighted prevalence of 2 (Mackin Index) is expected at four sites in Long Island Sound are given. This model allows the grower/manager to predict Dermo intensity in shellfish beds if field water temperature patterns are known. Such information can be used to select oyster growout beds and determine optimal time to harvest.

KEY WORDS: *Perkinsus marinus*, Dermo, temperature-disease course model, Long Island Sound

INTRODUCTION

Perkinsus marinus (commonly known as "Dermo"), a protozoan pathogen of uncertain phylogenetic affinities (Siddall et al. 1995) is now well established in Long Island Sound (Brousseau et al. 1994, Brousseau 1996, Ford 1996, Brousseau et al. 1998) and has been reported as far north as the Damariscotta River in Maine. This pathogen is a major cause of oyster mortality in the Gulf of Mexico and Chesapeake Bay. Its introduction to Long Island Sound, the third largest producer of commercial oysters in the U.S., has prompted efforts to develop management strategies and husbandry protocols to help control the spread of this disease.

The influence of temperature on the activity of *Perkinsus marinus* is well documented. Temperature affects multiplication rates, virulence (Andrews 1988) and zoosporulation of the parasite (Chu and Greene 1989), and disease intensity in the host increases with increasing temperature (Chu and LaPeyre 1993). Temperature is also believed to be the most important factor affecting the geographic distribution and seasonal cycle of this pathogen (Andrews 1988, Andrews and Ray 1988, Crosby and Roberts 1990, Soniat and Gauthier 1989).

Modeling studies also point to the importance of temperature in the development and maintenance of *Perkinsus marinus* infections. Simulations have shown that the timing and duration of long-term climatic changes are important in determining levels of infection in diseased (coinfection by MSX and Dermo) oyster populations (Powell et al. 1992); whereas, the results of Hofmann et al. (1995) suggest that temperature is the primary factor regulating the parasite in the Gulf of Mexico.

Soniat and Kortright (1998) recently developed a model to estimate the time to a critical level of *Perkinsus marinus* in eastern oysters using a long-term dataset of temperature, salinity, and parasite infection level. Their model indicates that both temperature and salinity are important variables in predicting Dermo progression in areas such as the Terrebonne estuary of Louisiana, where fluctuations in salinity are high and salinity levels often fall below 10 ppt. In high-salinity, oyster-producing regions such as Long Island Sound; however, it is likely that water temperature is the more important factor in controlling parasite proliferation.

This paper reports the results of a modeling study aimed at predicting the effect of short-term (seasonal) temperature changes on disease progression of "Dermo" in oyster populations from New York and southern New England. The annual cycle of *Perkinsus marinus* infections in oysters from six locations in Connecticut, Massachusetts, and New York is presented, and a predictive temperature-disease course model developed for wild and commercial oyster beds in Long Island Sound is described. A discussion of the usefulness of this model to oyster aquaculturists is also presented.

MATERIALS AND METHODS

Data Collection

Oysters (*Crassostrea virginica*) were collected twice a month from six locations in Connecticut, New York, and Massachusetts from January to December 1997 (Figure 1). Most samples contained 25 oysters; a total of 3,786 animals were studied. Sampling locations, site descriptions, sample sizes, and ages (juvenile vs. adult) of oysters sampled are provided in Table 1. Tissue diagnosis of *Perkinsus marinus* was done by culture of rectal and mantle tissue in fluid thioglycollate medium, as described by Ray (1954). Infections were scored for intensity of disease by use of the measure originally described by Mackin (1962) as the weighted incidence and later renamed weighted prevalence (Ragone and Burreson 1994). On the Mackin Index, scores of 0.5–1.0 indicate light infections, scores of 2.0–3.0 indicate moderate infections and scores of 4.0–5.0 are considered heavy.

Temperatures (°C) were monitored at each site using an Optic Stowaway™ Temperature Logger (Onset Computer Corp.). At intertidal sites, the recorder was attached to a stake driven into the flat, and at subtidal locations, it was suspended over the shellfish bed along a buoy system anchored to the bottom. As a backup against loss or failure, temperatures were also taken by hand several times a month. Periodic salinity measurements were taken to

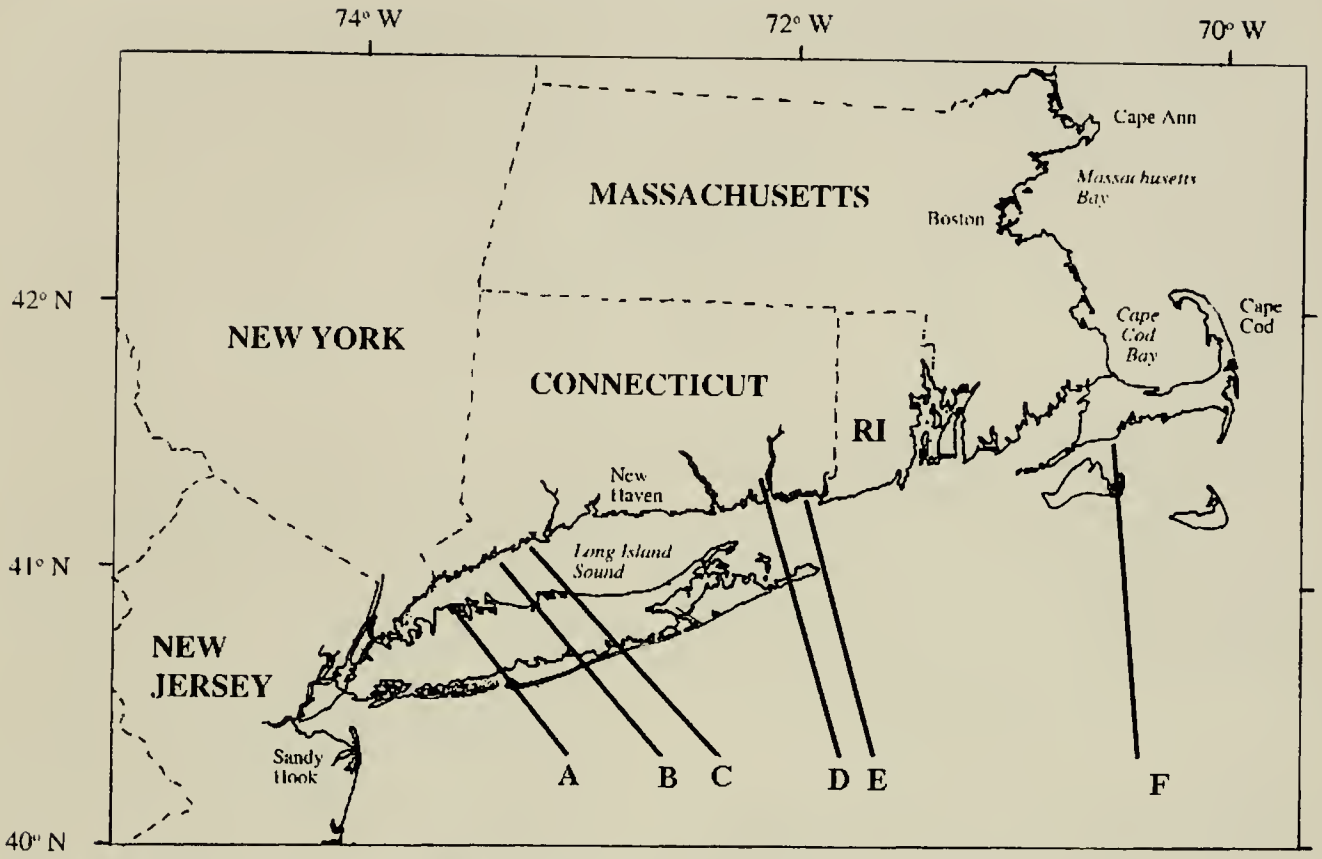

Figure 1. Map showing the locations of the six study sites used in this study: (A) Oyster Bay, NY, (B) Saugatuck River, Westport, CT, (C) Black Rock Harbor, Bridgeport, CT, (D) Thames River, Waterford, CT, (E) Mystic River, Stonington, CT, and (F) Cotuit, MA.

substantiate earlier reports that salinities at the study sites routinely run in the 20 to 30 ppt. range (Brousseau 1996, S. Ford pers. comm). They are shown in Table 2.

Mean Temperature Model

The mean temperature, T(x), for sampling day x can be modeled as a cyclic function

$$T(x) = \mu - \Delta \cos[c\ (x - x_{low})]$$

where μ is mean temperature for the year, Δ is one-half the range of mean temperatures (the amplitude), x_{low} is the day with the lowest mean temperature, and c is the constant needed to make the period equal to one year ($c = 2\pi/365.25$).

For x between the time of lowest mean temperature and highest

TABLE 1.

Age (adult vs. juvenile) and sampling location, number of samples collected, size of oysters sampled, site description (intertidal vs. subtidal), and type of oysters sampled (wild vs. cultivated).

Age and Sampling Location	Number of Samples	Shell Length (mm)	Mean Shell Length (mm)	Comments
Adult populations:				
Black Rock Harbor, Bridgeport, CT	25	38.5–133.7	70.4	1, 3
Mystic River, Stonington, CT	23	36.6–130.7	76.2	2, 4
Thames River, Waterford, CT	23	30.1–122.5	62.1	2, 4
Saugatuck River, Westport, CT	25	34.3–112.7	69.4	1, 3
Juvenile populations (1995/96 YOY):				
Black Rock Harbor, Bridgeport, CT	10	14.8–69.1	34.8	1, 3
Cotuit, MA	24	42.2–104.1	69.6	2, 4
Oyster Bay, NY	23	42.8–96.9	65.6	2, 4

1 = Intertidal sampling site.
2 = Subtidal sampling site.
3 = Wild population.
4 = Cultivated population.

TABLE 2.

Salinity measurements taken at the six study sites during 1997. (n = sample size).

Study Site	n	Mean ± SE (ppt)	Range (ppt)
Black Rock Harbor, Bridgeport, CT	128	23.1 ± 0.2	15.5–31.0
Mystic River, Stonington, CT	22	25.1 ± 0.5	18.1–28.0
Thames River, Waterford, CT	3	14.9 ± 4.9	5.4–21.7
Saugatuck River, Westport, CT	55	22.1 ± 0.4	11.5–27.0
Oyster Bay, NY	36	24.8 ± 0.2	22.0–26.0
Cotuit, MA	7	26.5 ± 0.3	25.0–27.0

mean temperature, the cumulative temperature, CT(x), is the area under the temperature curve from time x_{low} to time x:

$$CT(x) = \mu\,(x - x_{low}) - \Delta \sin[c\,(x - x_{low})]/c.$$

Separate temperature models were developed for each site (Black Rock Harbor, n = 309; Cotuit, n = 93; Mystic River, n = 365; Oyster Bay, n = 146; Thames River, n = 365; Westport, n = 365). Weighted nonlinear least squares analysis was used to fit the parameters.

Temperature—Disease Course Prediction Model

A linear model was used to describe the relationship between weighted prevalence y and lagged cumulative temperature for sampling day x and site s:

$$y = a + b\,CT_s\,(x - lag).$$

In this formula, $CT_s\,(x - lag)$ is the area under the mean temperature curve for site s from the time of lowest mean temperature to time x minus the lag. Cumulative temperature is used as an estimate of cumulative harm; parasite proliferation is assumed to be a function of ambient water temperature patterns at each site.

Samples with sampling day on or after the mean low temperature day for the site formed the working set for the analyses. A total of 87 samples were used (Bridgeport, n = 23; Mystic River, n = 20; Thames River, n = 21; Westport, n = 23). The lag was chosen to maximize the correlation between weighted prevalence and lagged cumulative temperature. The slope and intercept were then estimated using linear least squares. The model with best overall fit was chosen. Bootstrap analysis using 1,000 resamples was used to estimate the sensitivity of the choice of lag time in the model (Efron and Tibshirani 1993).

Model Predictions

To make predictions, a weighted prevalence of 2.0 was selected as the parameter of interest since we considered it a critical stage in the progression of the disease. Andrews (1988) reported that some mortalities are likely to occur when the mean intensity for a population exceeds 1.0; however, severe mortalities (50–75%) are not expected until the wp reaches 3.0 (Ray and Chandler 1955, Mackin 1961, Mackin and Hopkins 1961). Site-specific temperature models were then used to obtain point estimates for the day with mean weighted prevalence of 2.0. A bootstrap analysis with 1000 resamples was used to construct 95% confidence intervals for the day with mean weighted prevalence of 2.0 at each site.

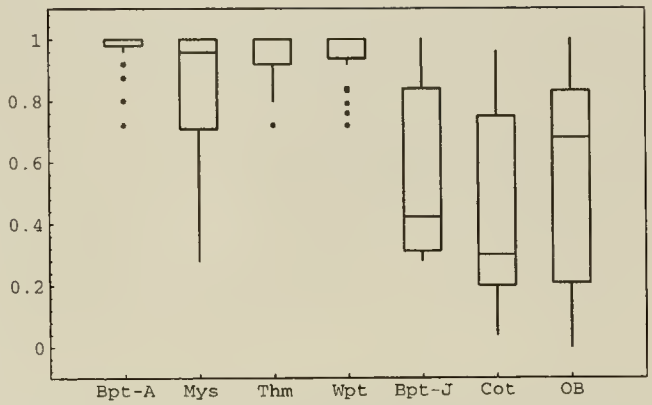

Figure 2. Side-by-side box plots of disease prevalence in juvenile and adult oysters. Adult populations in Bridgeport (n = 25 samples, median = 100% infected), Mystic River (n = 23 samples, median = 96% infected), Thames River (n = 23 samples, median = 100% infected), and Westport (n = 23 samples, median = 100% infected) and juvenile populations in Bridgeport (n = 10 samples, median = 42% infected), Cotuit (n = 24 samples, median = 30% infected), and Oyster Bay (n = 23 samples, median = 68% infected) are represented.

RESULTS

Descriptive Statistics

Disease prevalence in adult and juvenile oysters from all sites during 1997 is shown in Figure 2. Median values were between 96 and 100% for adult samples and between 30% and 68% for juvenile samples.

Distributions of weighted prevalences among sites are shown in Figure 3. The highest median weighted prevalence was found at the Saugatuck River site, followed by the Thames River and Black Rock Harbor sites. The median weighted prevalence was highest at sites where adult oysters were sampled (1.4–2.2); median weighted prevalence in juvenile oyster samples did not exceed 0.5. Distributions of proportions of oysters in all samples with intensity score of 2.0 or more on the Mackin scale is given in Figure 4. Median values for adult samples were between 32 and 60%; median values

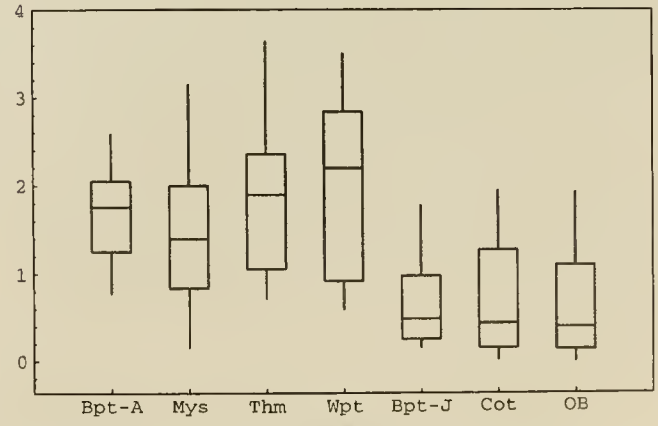

Figure 3. Side-by-side box plots of weighted prevalence in juvenile and adult oysters. Adult populations in Bridgeport (n = 25 samples, median = 1.76 wp), Mystic River (n = 23 samples, median = 1.40 wp), Thames River (n = 23 samples, median = 1.90 wp), and Westport (n = 25 samples, median = 2.20 wp) and juvenile populations in Bridgeport (n = 10 samples, median = 0.49 wp), Cotuit (n = 24 samples, median = 0.44 wp), and Oyster Bay (n = 23 samples, median = 0.40 wp) are represented.

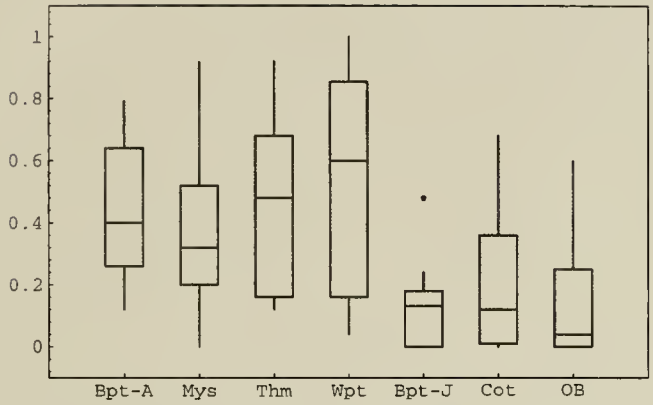

Figure 4. Side-by-side box plots of proportion with intensity score of 2.0 or more. Adult populations in Bridgeport (n = 25 samples, median = 40%), Mystic River (n = 23 samples, median = 32%), Thames River (n = 23 samples, median = 48%), and Westport (n = 25 samples, median = 60%) and juvenile populations in Bridgeport (n = 10 samples, median = 13%); Cotuit (n = 24 samples, median = 12%); and Oyster Bay (n = 23 samples, median = 4%) are represented.

for juvenile samples were between 4 and 13%. The largest proportions were observed at the Mystic River, Saugatuck River, and Thames River sites.

In adult oysters from Bridgeport, Thames River, Mystic River, Cotuit and Westport, weighted prevalence values increased dramatically during a 50-day period from the beginning of June to the middle of July. The proportion of individuals with infection intensities of 3.0 or higher also climbed during that time interval. In oysters from Oyster Bay, the shift from lower to higher weighted prevalences, and from a low to high proportion of moderate to severely diseased individuals also occurred over a 50-day period, but it happened later in the year (Figure 5). This result suggests a pattern of seasonal parasite proliferation for a population that begins in late spring or early summer and continues over a 7-week period, before reaching a plateau in mid- to late summer.

Temperature Models

Temperature model parameter estimates for each site are given in Table 3. Temperature patterns during 1997 were most similar at the Black Rock Harbor, Oyster Bay, Thames River and Westport sites, where mean maximum temperatures were between 21 and 23 °C. At Cotuit, on the other hand, mean temperatures peaked at 26 °C; whereas, in the Mystic River, mean temperatures reached a maximum of only 19 °C (Figure 6). The percentage of variation explained by the models ranged from 95 to 98%. The onset of seasonal proliferation of *Perkinsus marinus* at the study sites (Figure 5) coincides with approximate ambient water temperatures of 13 °C at the Mystic River, 16 °C at Bridgeport, Westport and the Thames River, and 20 °C at Cotuit and Oyster Bay (Figure 6).

Temperature—Disease Course Prediction Model

The prediction model with a lag time of 53 days gave the best over-all fit, explaining 45.1% of the variation in the data. The model equation

$$y = 0.94611 + 0.000899753\ CT_{x-53}$$

is based on 50 samples satisfying the condition that the sampling day minus 53 is between the mean low and mean high temperature days. Point and interval estimates for the day of the year with mean weighted prevalence of 2.0 are shown in Table 4.

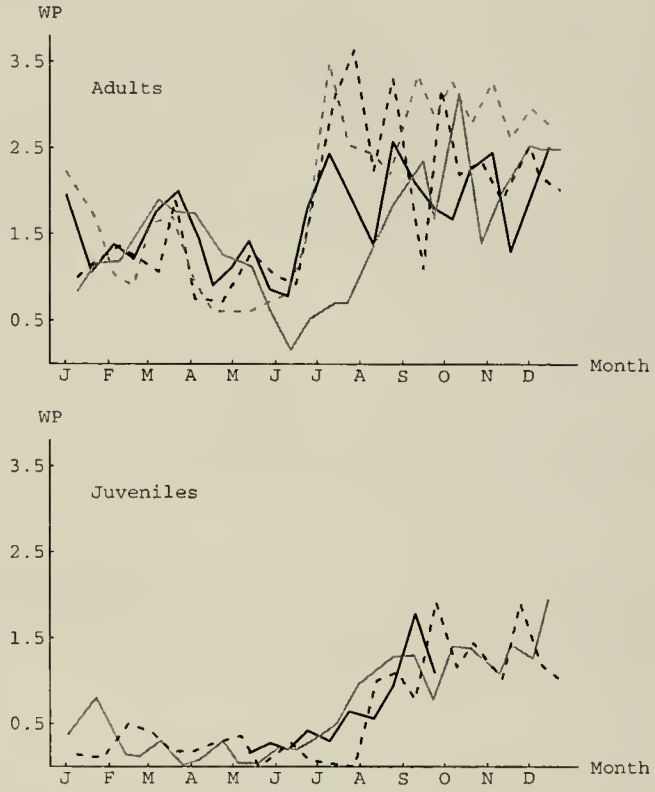

Figure 5. Graphs of weighted prevalence over time. Top plot: Bridgeport adults (n = 25 samples, solid black), Mystic River (n = 23 samples, solid gray), Thames River (n = 23 samples, dashed black), Westport (n = 25 samples, dashed gray). Bottom plot: Bridgeport juveniles (n = 10 samples, solid black), Cotuit (n = 24 samples, solid gray), Oyster Bay (n = 23 samples, dashed black).

For comparison, separate site-based models were developed and gave similar predictions.

DISCUSSION

Many of the characteristics of the Dermo epizootic in the northeast are similar to those described for epizootics in other areas. Disease prevalence is higher in adult oysters than in juveniles. Infection levels differ among size classes (ages); higher parasite burdens are found in adult oysters throughout the year. The lower infection intensities generally reported for juvenile oysters (Ray 1954) are believed to be the result of the relative growth rates of host and parasite (Mackin 1951, Hofmann et al. 1995). The plateau of high infection intensity seen in the northeast during the summer

TABLE 3.

Temperature model parameter estimates (μ: mean temperature; x_{low} = time of minimum average temperature; Δ = amplitude) for each study site.

Sampling Site	μ	x_{low}	Δ
Black Rock Harbor, Bridgeport, CT	12.19	35.16	10.59
Cotuit, MA	13.70	23.47	12.49
Mystic River, Stonington, CT	11.31	50.07	8.00
Oyster Bay, NY	11.40	40.14	11.67
Thames River, Waterford, CT	13.23	38.54	9.36
Saugatuck River, Westport, CT	11.33	38.68	10.49

has been reported for other infected populations as well (Crosby and Roberts 1990, Soniat 1985). The simulation study by Hofmann et al. (1995) has suggested that this buffering of infection intensity at levels of 3 to 4 on the Mackin scale may be attributable to two factors: (1) a decrease in parasite division rate at high parasite density; and (2) replacement of oysters that reach a lethal level of infection with less heavily infected oysters.

The northward spread of *Perkinsus marinus* into New England was not widely anticipated, because it had been viewed as a "warm-water" pathogen, which required minimal temperatures of 20 °C and extended periods of temperatures above 25 °C to establish an epizootic (Andrews 1988). Failure to predict the range expansion that has occurred may be attributable in part to lack of reliable water temperature data for oyster-growing areas. The most northerly site in this study, Cotuit, a shallow embayment on Cape Cod, experienced the highest mean water temperatures with temperatures consistently above 25 °C for over a month (Table 3; Figure 6), conditions similar to those reported for Delaware Bay (Ford 1996). The lowest mean water temperatures were recorded at Mystic, a deep-water site strongly affected by tidal exchange in and out of Long Island Sound. Site characteristics such as tidal exposure, water depth, tidal currents, and proximity to rivers or substantial freshwater inflow can be more important factors in determining the temperature characteristics of an area than its geographic location.

Infection levels in oyster populations began climbing when water temperatures reached 13–16 °C at the Bridgeport, Mystic River, Thames River, and Westport sites. This finding supports

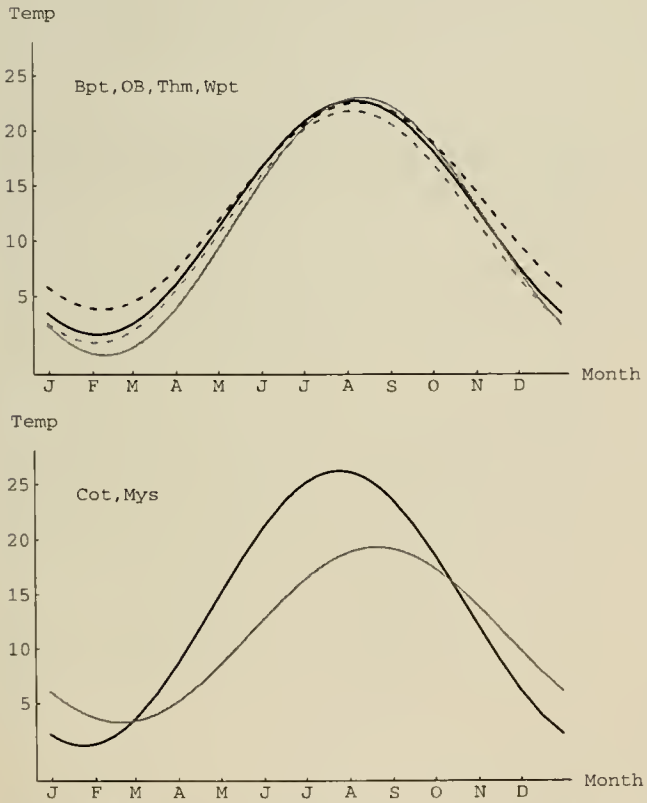

Figure 6. Mean temperature curves. Top plot: Bridgeport (maximum = 23 °C, solid black), Oyster Bay (maximum = 23 °C, solid gray), Thames River (maximum = 23 °C, dashed black), Westport (maximum = 22 °C, dashed gray). Bottom plot: Cotuit (maximum = 26 °C, solid black), Mystic River (maximum = 19 °C, solid gray).

TABLE 4.

Point and interval estimates for day of year with mean weighted prevalence of 2.0 determined for each study site.

Study Site	Day of Year	
	Point Estimate	Interval Estimate
Bridgeport	222	208, 237
Mystic River	237	221, 254
Thames River	215	200, 231
Westport	226	211, 241

earlier observations made for the Bridgeport population (Brousseau 1996). A later onset of parasite proliferation occurred among the juvenile oyster populations at Oyster Bay and Cotuit, when temperatures of 20 °C were reached, but the reason for the difference in timing is not known. Nonetheless, these results show a significantly different pattern of infection development from those reported in oysters from locations further south, where temperatures ≥20 °C are required for parasite proliferation (Andrews 1988). The reason for these observed differences in the temperature-time course of the disease are unknown, but possible hypotheses include: (1) the existence of a low temperature-adapted strain of the parasite (Bushek and Allen 1996, Dungan and Hamilton 1995) and/or (2) physiological differences in the immune systems of oysters from different geographic areas.

Soniat (1985) failed to find a correlation between water temperature and prevalence or intensity of *Perkinsus marinus*, but Crosby and Roberts (1990) found a statistically significant but weak correlation between water temperature and Dermo intensity. In a study that introduced lags into the relationship, Burreson and Calvo (1996) found significant correlation between water temperature and both prevalence and intensity of *Perkinsus marinus* in the Chesapeake Bay when lags of 2 to 4 months were used. The strongest relationship was with a 3-month lag; 46% of the variability in weighted prevalence and 39% of the variability in prevalence was explained.

The model developed for Long Island Sound showed the strongest relationship between cumulative temperature and *Perkinsus marinus* intensity when a lag time of 53 days was used. This result is similar to previous reports in the literature of significant correlations between temperature and parasite intensity when temperature was lagged by 60 days or more (Burreson and Ragone-Calvo 1996). It predicted that the oyster population from the Thames River would reach critical disease intensity levels (wp = 2) by late July/early August; whereas, similar intensity levels would not appear in the Mystic River until a month later. The eventual impact of the disease may depend on the time of the year when critical disease levels are attained. Very high oyster mortalities were experienced in the Thames River after mid-August 1997 (Janke pers. comm.) but no unusual mortalities were reported in the Mystic River during the year. The oyster mortality at the Thames River site may be attributable to high infection levels early in the season (Fig. 5) and higher mean temperatures during the year (Table 3). Any mortalities that may have occurred at the uncultivated sites (Bridgeport and Westport) went largely undocumented.

Water temperature is likely the most important single factor responsible for the establishment of *Perkinsus marinus* in the region of study, and although not controllable, knowledge of how the disease responds to differing environmental temperature patterns

can be helpful in managing oyster stocks in the face of disease pressure. Unlike most previous attempts to model the effects of environmental factors on the development and activity of *P. marinus* epizootics (Powell et al. 1992, Hofmann et al. 1995), this model has the advantage of being simple to use and having modest data requirements. It allows the grower to predict disease intensity in shellfish beds if field temperature patterns are known. The grower can then use this information in selecting oyster growout beds and determining optimal harvesting times for his product.

Admittedly, one drawback of using such a simple model for predicting parasite proliferation in the field is its failure to take into account additional factors that may affect local patterns of disease progression such as changing size frequency distributions within the population, yearly variations in food supply and annual changes in disease prevalence (Soniat et al. 1998). Also, this model was developed using only one year of data; it would benefit from additional tests over a longer time period to substantiate its general applicability. In spite of these shortcomings; however, the modeling approach presented here shows promise, and with further testing could prove to be a useful tool in industry efforts to minimize the impact of Dermo disease.

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OSMOTIC TOLERANCE AND VOLUME REGULATION IN *IN VITRO* CULTURES OF THE OYSTER PATHOGEN *PERKINSUS MARINUS*

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ABSTRACT Growth rate, cell size, osmotic tolerance, and volume regulation were examined in cells of *Perkinsus marinus* cultured in media of osmolalities ranging from 168 to 737 mOsm (6.5–27.0 ppt). Cells cultured at the low osmolalities of 168 and 256 mOsm (6.5 and 9.7 ppt) began log phase growth 4 days postsubculture, whereas cells cultured at the higher osmolalities 341, 433, and 737 mOsm (12.7, 16.0, and 27.0 ppt) began log phase growth 2 days postsubculture. During log phase growth, cells from the higher osmolalities 341, 433, and 737 mOsm had shorter doubling times than cells from the lower osmolalities 168 and 256 mOsm. During both log and stationary phase growth, the mean cell diameter of cells cultured at 168 mOsm was significantly greater than cells cultured at 341 and 737 mOsm; the mean diameters of cells cultured at 341 and 737 mOsm did not differ significantly from each other. *P. marinus* cells cultured in various osmolalities were exposed to artificial seawater treatments of 56–672 mOsm (2.5–24.7 ppt). After the hypoosmotic treatment of 56 mOsm, cells that had been cultured in medium of low osmolality, 168 mOsm, showed only 41% mortality whereas the cells from the 341-, 433-, and 737-mOsm culture groups experienced 100% mortality. During the hyperosmotic shock, all of the groups exhibited mortalities of less than 10%. In *P. marinus* cells cultured in medium of 737 mOsm and then placed in a 50% dilution, cell diameter increased 13%, which was a volume increase of 44.5%, but cells returned to baseline size (size before osmotic shock) within 5 minutes. *P. marinus* cells cultured at low osmolalities can withstand both hypo- and hyperosmotic stress and use volume-regulatory mechanisms during hypoosmotic stress. Results suggest that transferring infected oysters to low salinity will result in strains of *P. marinus* acclimated to low salinity that will be able to withstand periodic events of extremely low salinity.

KEY WORDS: Osmotic tolerance, volume regulation, *Perkinsus marinus*

INTRODUCTION

Perkinsus marinus, a parasite of the eastern oyster, *Crassostrea virginica* (Gmelin), was first reported in the Gulf of Mexico (Mackin et al. 1950) but is now observed in *C. virginica* along the Atlantic west coast from Maine to Florida and in the Gulf of Mexico from Florida to Mexico (Andrews and Hewatt 1957, Mackin 1962, Burreson et al. 1994a). Since the 1950s and especially since 1986, *P. marinus* has been a major cause of mortality in the eastern oyster in the Chesapeake Bay (Burreson and Ragone Calvo 1996).

The eastern oyster, *C. virginica*, is an osmoconformer, but the osmotic tolerances of the parasites *Haplosporidium nelsoni* (MSX) and *P. marinus* living within the oyster are not clearly defined (Ford and Haskin 1988). Salinity is believed to be an important environmental factor that regulates the prevalence and intensity of *H. nelsoni* and *P. marinus*. These two common oyster parasites, however, appear to have differing tolerances to hypoosmotic conditions. Ford (1985) reported a reduced prevalence of *H. nelsoni* in oysters in salinities lower than 15 ppt. Ford and Haskin (1988) showed that some killing of *H. nelsoni* occurred at 15 ppt with maximum elimination at 9 ppt, suggesting that the pathogen is physiologically unable to tolerate low salinities. *P. marinus* tolerates salinities lower than 12 ppt, but the mechanisms that allow survival in low-salinity environments have not been clearly defined (Ragone and Burreson 1993, Burreson and Ragone Calvo 1996). Studies have shown that low salinity has a retarding effect on *P. marinus* development (Ray 1954, Mackin 1962, Soniat 1985, Burreson and Ragone Calvo 1996). In addition, it has been reported that infection intensity of *P. marinus* is positively correlated with temperature and salinity (Soniat 1985, Soniat and Gauthier 1989, Crosby and Roberts 1990, Burreson and Ragone Calvo 1996). An *in vivo* study of oysters infected with *P. marinus* determined the critical salinity range for pathogenicity to be between 9 and 12 ppt, and that *P. marinus* was less virulent below 9 ppt (Ragone and Burreson 1993). Also, the study reported that lower salinities (6 and 9 ppt) delayed disease development, whereas infections at higher salinities (12 and 20 ppt) increased in intensity and resulted in higher levels of oyster mortality.

Despite these findings, little is known about the osmotic tolerance of *P. marinus* when faced with hypo- and hyperosmotic stress. Studies with both free-living and parasitic protozoa have shown that many protozoa have the ability to adjust their cell volumes when faced with external osmotic changes (Kaneshiro et al. 1969, Da Silva and Roitman 1982, Geoffrion and Larochelle 1984, Ahmad and Hellebust 1986, André et al. 1988, Cronkite and Pierce 1989, Hellebust et al. 1989, Darling and Blum 1990, Darling et al. 1990). Similarly, *P. marinus* may also utilize physiological mechanisms to adjust to its changing osmotic environment. Only one previous study has been conducted on the osmotic tolerance of *P. marinus* in the absence of host influences (Burreson

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et al. 1994b). This study reported that cells cultured at 22 ppt and placed in extreme low-salinity treatments of 0 and 3 ppt had higher than 90% mortality. As a continuation of this work, we investigated the osmotic tolerances and volume-regulatory abilities of *P. marinus* cells, which have been cultured in a range of osmotic conditions (168–737 mOsm or 6.5–27.0 ppt) and exposed to various osmotic treatments (56–672 mOsm or 2.5–24.7 ppt).

MATERIALS AND METHODS

In Vitro Cultures of P. marinus

Cultures of *P. marinus* were maintained in Jeronie La Peyre-Oyster Disease Research Program-1 (JL-ODRD-1) medium (La Peyre et al. 1993) (approximately 737 mOsm or 27.0 ppt) without bovine serum albumin (BSA) in a humid atmosphere at 28 °C in a 5.0% CO_2 incubator. Cells from the BSA-free acclimated cultures were transferred from 737-mOsm culture medium into 168, 256, 341, and 433 mOsm (approximately 6.5, 9.7, 12.7, and 16.0 ppt) media in a gradual procedure in which cells from 737 mOsm were placed into 433, 433 into 341 mOsm, etc., with the stepwise transfer occurring every 3 days. For culture maintenance, subculturing occurred every 2–4 wk. Cultures were seeded at a density of 5×10^6 cells per 5 mL of medium for all experiments, and during these experiments, subculturing occurred every 2 wk. Growth curves for the groups cultured at 168, 256, 341, 433, and 737 mOsm were determined by obtaining cell counts with a hemacytometer (Fisher Scientific) every day for 12 days starting the day after subculture to determine the time period of log phase growth. The growth rate study used cells approximately 20 generations (about 1 y) descending from the original cultures that were first acclimated to the different osmolalities. A generation is defined as one subculture. Cell size experiments used cells that were approximately 25–30 generations descended from the acclimated cultures. The osmotic tolerance experiments used cultures that were 7–10 generations descended from the original groups acclimated to the different osmolalities.

Culture Media

The cell culture medium used for the *P. marinus* cultures was the JL-ODRP-1 (La Peyre et al. 1993) without BSA. Media (100 mL) equivalent to 168, 256, 341, 433, and 737 mOsm were prepared before each subculture for the different culture groups following methods described by La Peyre et al. (1993). In addition to the reported constituents, the culture medium, depending on the desired osmolality (168, 256, 341, 433, and 737 mOsm), also included basal synthetic sea salts (0.3, 0.6, 0.9, 1.2, or 2.2 g), 0.2 g $NaHCO_3$, and KCl (0.0061, 0.0079, 0.0097, 0.0115, or 0.0177 g) dissolved in 91.5 mL tissue culture-grade water.

Cell Sizes of Cultured Cells

Cell diameters of the various *P. marinus* groups in both log and stationary growth phase were measured by using the NIH Image Analysis (Version 1.56) Macintosh computer program for particle size analysis and the MediaGrabber Macintosh program with RasterOps video digitizer board to capture live microscopic images from an inverted Zeiss light microscope (40× objective used in all of the cell size experiments). Cell measurement techniques with image analysis were based on methods described by Weeks and Richards (1993).

Baseline measurements were initially conducted to determine whether the groups cultured in media with osmolalities of 168, 341, and 737 mOsm varied in cell size. For log phase growth size distributions, cells cultured in 168-, 341-, and 737-mOsm media were harvested 6 days after subcultured and transferred to 15-mL microcentrifuge tubes. Each group of cells was declumped by repeatedly withdrawing the cells and passing them through a 3-mL syringe (25G 7/8-inch hypodermic needle). Cells were centrifuged at 800 *g* for 15 minutes, the medium decanted, and the cells resuspended in 10 mL of isotonic seawater. Seawater solutions (173, 365, and 740 mOsm or 6.7, 13.6, and 27.1 ppt) that were isotonic to the culture medium of each group consisted of 97.5 mL tissue culture-grade water, basal synthetic sea salts (0.45, 1.05, or 2.35 g), 0.2 g $NaHCO_3$, KCl (0.0061, 0.0097, or 0.0777 g), and 2.5 mL HEPES buffer (original concentration = 239.02 mg/mL). After resuspending the cell pellets in the isotonic artificial seawater solutions, cell solutions were stirred with a vortex mixer (Fisher Scientific), and a 10-μL sample was withdrawn from each group for cell counts using a hemacycometer. Volumes containing 1×10^5 cells from each group (168, 341, and 737 mOsm) were calculated, and these cell solutions were added to three different cell wells (three wells per group) in a cell well plate. From each well of each of the three groups, three to four images were captured. The number of cells per image ranged from approximately 40 to 70 cells. Clumped cells that could not be easily distinguished were excluded. This cell sizing protocol was also followed to measure cells cultured at 168, 341, and 737 mOsm in stationary phase growth (2 wk after subculture). Mean cell diameters were calculated for the culture groups from both log and stationary growth phase, and the relationship between culture medium osmolality and cell diameter was examined by a one-way analysis of variance. Significant differences between the groups cultured at the three different osmolalities were determined by using the Scheffé *post hoc* multiple comparison test.

Osmotic Tolerance

Buffered artificial seawater (ASW) treatment solutions of 56, 135, 222, 305, 386, and 672 mOsm (approximately 2.5, 5.3, 8.5, 11.4, 14.4, and 24.7 ppt, respectively) were prepared by dissolving synthetic basal salts (Sigma Chemical Co.) (0.0, 0.3, 0.6, 0.9, 1.2, or 2.2 g), 0.1176 g $NaHCO_3$, KCl (0.0014, 0.0044, 0.0061, 0.0078, 0.0097, or 0.0156 g), and 2.5 mL HEPES buffer (original concentration = 239.02 mg/mL) in 97.5 mL of tissue culture-grade water. After adding these constituents, the mixtures were adjusted to a pH of 7.5 and then filter sterilized. All of the treatment solutions, the BSA-free media for the culture groups, and the isotonic seawater solutions (used for cell size experiments) were analyzed on a vapor pressure osmometer (Wescor) to determine osmolalities. Cell density by hemacytometer and cell viability of the *P. marinus* cultures were assessed in each culture group (168, 256, 341, 433, and 737 mOsm). To determine cell viability, a 100-μL subsample was placed in a microcentrifuge tube and 10 μl of 0.05% neutral red stain added. After 10 min, two 10-μl aliquots were placed on the hemacytometer. Both live (stained) and dead (unstained) cells were counted for at least 200 cells. From each group, 2.0×10^6 cells were added to sterile 15-mL centrifuge tubes and the volumes raised to 7 mL with the treatment ASW at the osmolality equivalent to the medium osmolality. Then, 1 mL of each of these cell suspensions was centrifuged at 470 *g* for 5 min. The supernatant was decanted and the pellet resuspended in 1 mL of each of the treatment osmolalities (ASW) in a 24-well tissue culture plate. Thus, *P. marinus* cells cultured in media of 168, 256, 341, 433, and

737 mOsm were placed in ASW treatment osmolalities of 56, 135, 222, 305, 386, and 672 mOsm for 24 hours in 24-well microtiter plates at 28 °C in an incubator without CO_2. After the 24-hour incubation, 100 µL of neutral red was added and gently mixed with a pipette tip. Mortality was assessed by counting live and dead cells in two to three random grid fields with an inverted light microscope (Zeiss) and a 10 × 10-mm ocular micrometer grid. The experiment was repeated three times. Logistic regression analyses with SAS procedure Catmod were utilized to examine the response of the population (culture group) to the treatment osmolality and to calculate predicted mortalities (which describe the response of each population) with 95% confidence intervals for each of the culture groups at each treatment osmolality. A logistic regression model was chosen to represent the binary response of mortality (live *versus* dead). In addition, the actual live and dead cell counts were used for calculating percent mortalities and for an analysis that compares proportions from independent samples (Fleiss 1981).

Cell Size after Hypoosmotic Shock

Cell diameter changes following a hypoosmotic shock were measured with the MediaGrabber and NIH Image Analysis systems. Cells cultured in medium of 737 mOsm were harvested 2–3 wk after subculture, declumped with a 3-mL syringe (25G 7/8-inch hypodermic needle), and centrifuged at 800 *g* for 15 min. The medium was decanted, and isotonic seawater was added to obtain a volume of 10 mL. Cell density was determined with a hemacytometer, and a volume containing 1×10^5 cells was added to a cell well. A volume of 173 mOsm ASW was added to the well to result in a 50% dilution of the original seawater solution. Before adding this calculated volume of the hypoosmotic shock solution, an image was captured to represent time 0. Ten to twenty seconds after the 50% dilution, a second image was captured as time 1. Images were then captured at 1, 3, 5, 7, 10, 12, 15, 20, 30, 45, and 60 min after dilution. The same cells from the same plane were eaptured as images, and thus, the same population experiencing the shock was represented. These images were analyzed with the NIH Image Analysis system to determine cell diameters at each time interval. The experiment was repeated five times. The first experiment used cells 19 days postsubculture. The second experiment used cells from a different culture 18 days postsubculture and included time points of 0 and 10–20 sec, and 1, 3, 5, 10, 15, 20, and 30 min. The third, fourth, and fifth experiments used cells 20 days postsubculture and were performed consecutively on the same day with cells from the same flask. Experiments 3, 4, and 5 included images captured at 10–20 sec and 1, 3, 5, 10, 15, 20, and 30 min. Cell viability was assessed with the vital stain neutral red before the shock and 30 min after adding the shock solution. The control experiment used cells 21 days postsubculture and followed the protocol described above without adding the shock solution; images were obtained at 0, 1, 3, 5, 10, 15, 20, and 30 min. Cell sizes after hypoosmotic shock were analyzed with the nonparametrie Kruskall-Wallis test to first examine the effect of each experiment. To separate out the significant effect of each experiment but still look at the results of all trials together to examine the overall effect of the treatment osmolality on cell size, a mean center standardization was used by subtracting the mean cell diameter (total mean diameter for all time points within each experiment) from each data point. A second Kruskall-Wallis test was run on the standardized data to examine whether each experiment continued to have a significant effect on cell diameter. The effect of the experiment was no longer significant, and the experiments were pooled. A third Kruskall-Wallis test was used to determine whether time had a significant effect on cell diameter. Lastly, the Tukey-Kramer multiple comparison *post hoc* analysis was implemented to determine at which time points the mean cell diameters were significantly different from each other. An unpaired *t*-test was used to determine whether there was a significant difference between the standardized control diameters and the standardized replicate diameters (experiments pooled) both before the shock and 1 min after the shock.

RESULTS

Growth Rate

The results of the growth rate study indicated that log phase growth began approximately 2 days postsubculture for *P. marinus* cells cultured in 341, 433, and 737 mOsm (12.7, 16.0, and 27.0 ppt) media. Cultures from the 168 and 256 mOsm (6.5 and 9.7 ppt) media began log phase growth approximately 4 days postsubculture (Fig. 1). The groups cultured at the higher osmolalities of 341, 433, and 737 mOsm had shorter doubling times compared with the groups cultured at the low osmolalities of 168 and 256 mOsm. For the 168-mOsm cells, 35.2 h were required for one doubling and 35.7 h for the 256-mOsm cells. For the higher osmolality cells from 341, 433, and 737 mOsm, one doubling required 22.8, 25.9, and 24.4 h, respectively.

Cell Sizes of Cultured Cells

During log phase growth, the mean diameters (± standard error) of *P. marinus* cells cultured in media of 168, 341, and 737 mOsm were 11.8 (±0.191), 9.6 (±0.108), and 9.2 (±0.106) µm, respectively. The effect of culture medium osmolality on cell diameter

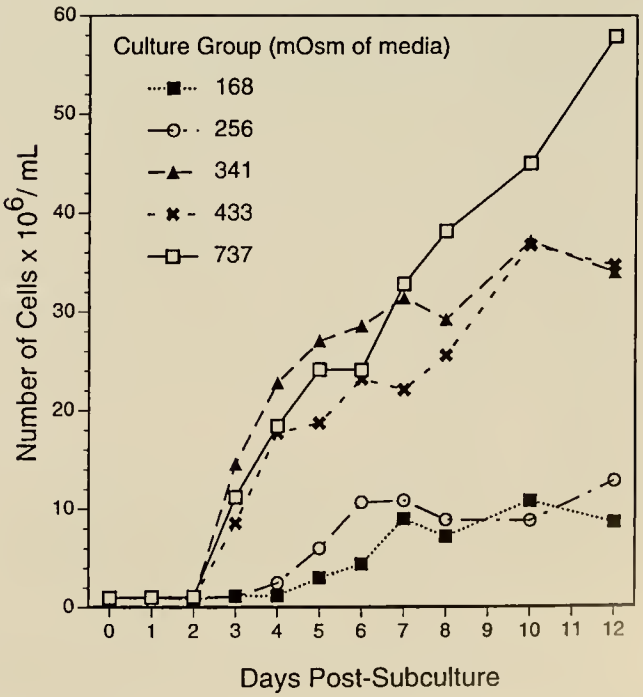

Figure 1. Growth curve of *P. marinus* cells cultured in media of 168, 256, 341, 433, and 737 mOsm (6.5, 9.7, 12.7, 16.0, and 27.0 ppt).

was statistically significant ($P = 0.0001$). Cells cultured at 168 mOsm were significantly larger than cells at either 341 ($P < 0.0001$) or 737 mOsm ($P < 0.0001$). The differences in diameter between cells at 341 and 737 mOsm were not significant ($P = 0.1565$). The mean diameters of stationary phase *P. marinus* cells cultured at 168, 341, and 737 mOsm were 8.4 (±0.165), 4.7 (±0.070), and 5.1 (±0.093) μm, respectively. As observed with cells from log phase growth, the effect of culture medium osmolality on cell size was statistically significant ($P < 0.0001$). Cells cultured at 168 mOsm were significantly larger than cells at either 341 ($P < 0.0001$) or 737 mOsm ($P < 0.0001$), whereas the difference in cell diameter between the 341- and 737-mOsm groups was only significant at the 5% level of significance ($P = 0.021$).

Osmotic Tolerance

Before osmotic shock, the mean viabilities of the *P. marinus* cells cultured at 168, 256, 341, 433, and 737 mOsm were 88.2%, 96.2%, 99.1%, 99.3%, and 98.8%, respectively. After hypoosmotic treatments, the percent mortality was lower in groups that were cultured in low-osmolality media than in groups from higher osmolalities (Fig. 2). For example, in the extreme hypoosmotic shock of 56 mOsm (2.5 ppt), mortality was 41% in cells cultured at an osmolality of 168 mOsm but was 100% in cells that were cultured at 737 mOsm. Conversely, in the hyperosmotic shock of 672 mOsm (24.7 ppt), groups that had been cultured at low osmolalities as well as high osmolalities all experienced mortalities of less than 10% (Fig. 2). A logistic regression analysis showed that a significant relationship existed between treatment osmolality as a function of mortality ($P < 0.001$). A comparison of proportions from independent samples test showed that the mortality response of the 168-mOsm group was significantly different ($P < 0.001$) from the mortality observed for the 737-mOsm culture group at the 56-mOsm treatment. Predicted mortalities determined from a logistic regression analysis indicated that in low-osmolality treatments, groups cultured at 168 and 256 mOsm have lower mortalities than the groups that had been cultured at 341, 433, and 737 mOsm.

Cell Size after Hypoosmotic Shock

Cell viability was not affected by the 50% dilution; the results of a viability test indicated a 99% viability before the dilution (time 0) and 97% viability 30 min after the dilution. From the first nonparametric analysis, it was difficult to examine the effect of osmolality on cell size because of variability between experiments and variability between experimental conditions. After implementing a mean center standardization, however, nonparametric analysis indicated that each experiment did not have a significant effect on cell diameter ($P = 0.8976$). Therefore, the results from each experiment could then be pooled. The overall response to the 50% hypoosmotic shock was an initial swelling followed by a return to baseline size (Fig. 3). When placed in the 50% dilution treatment, *P. marinus* cells that were cultured at 737 mOsm experienced an initial swelling between 0 and 30 sec after hypoosmotic shock. Cells swelled and returned to baseline size within about 5 min. The mean diameter change during swelling was 0.7 μm. The initial mean cell diameter was 5.7 μm, and thus, the percent diameter increase during initial swelling was approximately 13%, which was a 44.5% change in cell volume. The nonparametric analysis on the pooled, standardized data showed that time had a significant effect on cell diameter, with a tied P-value of <0.0001. The *post hoc* multiple comparison analysis with a $P < 0.05$ level of significance indicated that significant differences existed between the following time points: 0 and <30 sec, <30 sec and 5 min, <30 sec and 15 min, <30 sec and 20 min, and <30 sec and 30 min. The unpaired t-test showed no significant difference between the mean diameters of the control group and the experimental groups (all experiments pooled) at time 0 ($P = 0.2931$), but there was a significant difference at a significance level of $P < 0.05$ between the control group and the experimental groups 1 min after the shock ($P = 0.0022$).

DISCUSSION

Continuous cultures of *P. marinus* can be maintained in low-osmolality environments (as low as 168 mOsm or 6.5 ppt). Fur-

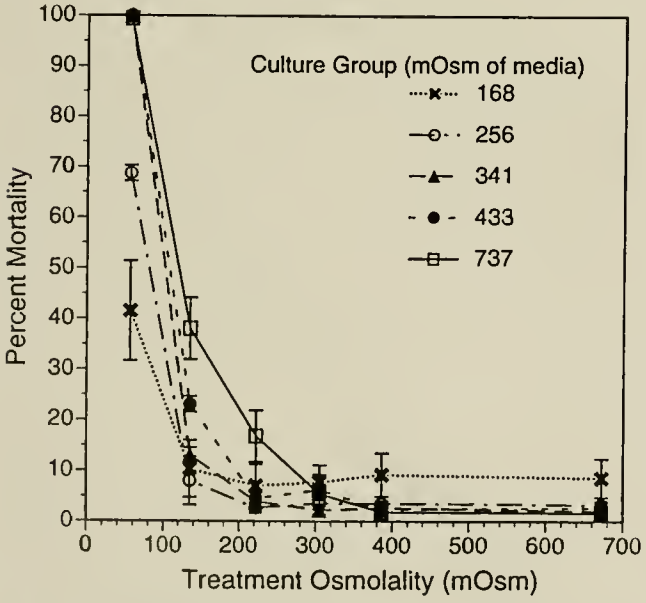

Figure 2. Percent mortality of *P. marinus* cells cultured in media of 168, 256, 341, 433, and 737 mOsm (6.5, 9.7, 12.7, 16.0, and 27.0 ppt) and placed in treatment osmolalities of 56, 135, 222, 305, 386, and 672 mOsm (2.5, 5.3, 8.5, 11.4, 14.4, and 24.7 ppt) for 24 h. Error bars = standard error.

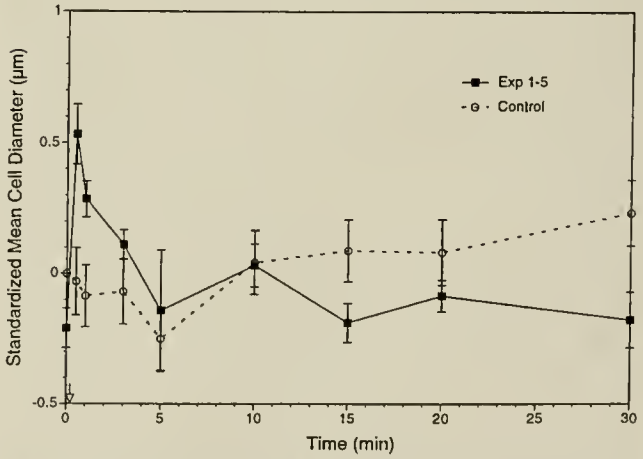

Figure 3. Standardized mean cell diameter (μm) of *P. marinus* cells cultured at 737 mOsm (27.0 ppt) and placed in 50% hypoosmotic shock (arrow) with experiments 1–5 pooled and the control experiment. Arrow indicates actual time of shock; time represents time after shock. Error bars = standard error.

thermore, cells maintained in osmolalities ranging from 168 to 737 mOsm (6.5–27.0 ppt) are tolerant of hypo- and hyperosmotic conditions in the treatment range of 222–672 mOsm (8.5–24.7 ppt). Cells cultured at low osmolalities can also withstand extreme low osmolalities such as 56 mOsm (2.5 ppt) for at least 24 hours. Thus, these experiments have shown that cultured cells of *P. marinus* can survive both hypo- and hyperosmotic stress. During hypoosmotic stress, cells increased in diameter, followed quickly by a return to baseline size (size before osmotic shock), which indicates a volume-regulatory response. This response helps explain why *P. marinus* continues to persist in the Chesapeake Bay despite periods of low salinity that occur during times of high rainfall and runoff into the tributaries.

The growth rate study showed that *P. marinus* cells that were cultured at osmolalities of 341, 433, and 737 mOsm (12.7, 16.0, and 27.0 ppt) reached log phase growth before cells cultured at lower osmolalities of 168 and 256 mOsm (6.5 and 9.7 ppt). In addition, cells from higher osmolalities had greater rates of multiplication (shorter doubling time) during log phase than cells cultured in low osmolalities. These results correspond to a study with trypanosome cultures that showed that media of high osmolality supported greater multiplication rates than low-osmolality media (Da Silva and Roitman 1982).

The osmotic tolerance study indicated that *P. marinus* cells cultured at low osmolalities experienced reduced mortality when placed in extreme hypoosmotic conditions when compared with the groups cultured at higher osmolalities. Because the cells were already acclimated to the stress of a low-osmolality environment, they were able to withstand an extreme low osmolality of 56 mOsm better than cells cultured at much higher osmolalities. Approximately 60% of the 168-mOsm cultured cells survived the extreme low osmolality of 56 mOsm for at least 24 hours. In this study, all of the culture groups had low mortalities (<10%) after hyperosmotic stress. Consequently, *P. marinus* was more tolerant of hyper- than hypoosmotic shock.

This research showed that the stressor did not seem to be the magnitude of the shock, but instead the type of shock (hypo- or hyperosmotic) and the actual osmolality of the challenge treatment. For example, the 737-mOsm cells placed into 222-mOsm treatment (a difference of 515 mOsm) had much higher mortality than cells from 168-mOsm placed into 672-mOsm treatment (a difference of 504 mOsm). Although the magnitude of the shock was about the same, the hypoosmotic rather than the hyperosmotic environment was more stressful, as indicated by higher mortality levels. In addition to the type of stress, the actual osmolality of the stress affected the level of mortality. For instance, cells acclimated to 737 mOsm and placed into 386-mOsm treatment (a difference of 351 mOsm) had much lower mortality (<10%) than cells from 433 mOsm placed into 56-mOsm treatment (a difference of 377 mOsm), which resulted in 100% mortality. Although the magnitude of both of the hypoosmotic shocks was similar, mortality was higher in the treatment with the lowest absolute osmolality, indicating the cells may have a threshold osmolality level needed for survival.

The results from the osmotic tolerance experiment differ from the study by Burreson et al. (1994b), which reported much higher mortality levels in cells acclimated to 737 mOsm and placed in the same hypoosmotic treatments. The study by Burreson et al. (1994b) showed greater than 60% mortality for cells acclimated to 737 mOsm and placed in treatments of 136 mOsm and 213 mOsm, whereas this study reports 15–40% mortality in the same low-osmotic treatments. One difference is that Burreson et al. (1994b) used *P. marinus* cells cultured in medium with BSA (known as JL-ODRP-1 media), whereas this study used cells cultured in BSA-free medium. However, comparative experiments with cells acclimated to either medium with BSA or BSA-free medium showed no significant difference between the effects of the two types of media on osmotic tolerance. Other factors that may have contributed to the differences in mortality between this experiment and the previous one include reported differences in experimental design such as the age of the cells (numbers of subcultures since isolation and initiation), growth phase of the cells, and type of incubator used (CO_2 or without CO_2). For example, the cells in the previous study were transferred to an incubator without CO_2 for 1 week before use, whereas cells in our experiment were in an environment without CO_2 for only 1 day. The prolonged exposure to an environment without CO_2 may have stressed the cells in the previous study, making them more susceptible to mortality after osmotic shock. Growth rates are reduced in cultures that have been transferred to an incubator without CO_2 when compared with cultures maintained in a 5.0% CO_2 incubator (La Peyre, personal observation).

Cells cultured at the low osmolality of 168 mOsm were significantly larger than cells cultured at the high osmolalities of 341 and 737 mOsm during both log and stationary growth phases. The cells cultured at the high osmolalities of 341 and 737 mOsm, however, were not significantly different from each other in size. The difference in cell size may be due to increased water content required to match the low osmolality of the dilute external medium. A study with red coelomocytes of the euryhaline polychaete *Glycera dibranchiata* showed cells acclimated to a lower osmolality had a higher "body-wall-tissue water content" and greater cellular volume than cells acclimated to a higher osmolality (Costa et al. 1980). An experiment with the amoeba *Acanthamoeba castellanii* indicated that the amount of intracellular water increased when cells were placed in a severe hypoosmotic shock (Geoffrion and Larochelle 1984). Similarly, the gradual acclimation of the *P. marinus* cells from high- to low-osmolality media when developing low-osmolality cultures may have caused an increase in size as water initially diffused into the cells, and the cells cultured in the low osmolality may not have been able to completely return to baseline size during volume regulation because of the stress of the prolonged hypoosmotic environment. Cells must maintain certain levels of metabolites to survive the stress of a low-osmolality environment. These levels of solutes attract water molecules because of simple diffusion, and therefore, an increased intracellular water content results. Studies on the erythrocytes of the bivalve *Noetia ponderosa* (Amende and Pierce 1980, Smith and Pierce 1987) and a report on the euryhaline ciliate *Paramecium calkinsi* (Cronkite and Pierce 1989) indicated that cells may not always completely return to baseline (size before osmotic shock) after volume regulation.

Alternatively, the difference in cell size of the groups cultured at low *versus* high osmolalities may be due to a difference in life stages of the groups that were measured. Cells of *P. marinus* divide by schizogony with a cell increasing in size, acquiring a vacuole, and then releasing several daughter cells (La Peyre and Faisal 1997). This process could have been occurring with some of the cells from the low-osmolality cultures during the cell-size experiment, as both small cells and large cells with smaller cells inside them were observed, whereas the groups measured at higher osmolalities mainly consisted of small cells. Thus, because the

low- and high-osmolality groups had different growth rates, they may not have been at the same growth stage when their cell diameters were measured, which could account for the differences in size between the groups. The cells from higher osmolalities were not observed as a large parent cell dividing into several smaller cells, but instead, one cell often appeared to divide into two (data not shown). *P. marinus* cells with high growth rates appear to divide as one small cell dividing into two cells (La Peyre 1996). The cells at the low osmolality, however, may be larger in size even before schizogony because of an increased internal water content. Further studies examining the relationship between medium osmolality, *P. marinus* growth stage, and cell size would be useful in understanding the role of osmolality in *P. marinus* growth and survival.

During the short-term hypoosmotic stress experiment in this study, *P. marinus* cells followed a typical cell volume response that is observed in other organisms by experiencing an initial swelling and then shrinkage back toward baseline (Costa et al. 1980, Smith and Pierce 1987, Cronkite and Pierce 1989, Darling et al. 1990). The results indicate that *P. marinus* cells do not resist swelling during sudden or extreme external osmolality changes. The size at the maximum swell was significantly different from the initial baseline and the acclimated sizes. The erythrocytes of the clam *N. ponderosa* exhibited a similar pattern when cells acclimated to 935 mOsm were placed in a hypoosmotic shock of 560 mOsm; the cells swelled, thereby increasing their volume by 50% within 5 min followed by a gradual return toward baseline (Smith and Pierce 1987). Because the cells in this study did swell and return to baseline size, the results suggest that *P. marinus* regulates the intracellular osmotic concentration to regulate cell volume during changing external osmolalities. The results reported here along with other studies by our laboratory (data not shown) and by Paynter et al. (1997) on intracellular osmolytes (i.e., free amino acids) used by *P. marinus* indicate that *P. marinus* cells utilize volume-regulatory mechanisms to compensate for osmotic changes in the external medium. These mechanisms enabled cells in this study to survive a 50% dilution of the external medium. But to better describe the specific volume-regulatory mechanisms used by *P. marinus*, current studies are focusing on measuring the levels of intracellular inorganic ions and organic molecules before, during, and after osmotic shock to determine their role in volume regulation.

The results of these experiments help explain why *P. marinus* continues to persist in the upper portions of the Chesapeake Bay tributaries despite periods of low salinities. Periodic increases in stream flow causing lowered salinities have not greatly affected the abundance of *P. marinus* in Chesapeake Bay tributaries (Burreson and Ragone Calvo 1994, Ragone Calvo and Burreson 1995). The fact that low salinities have not eradicated the pathogen from these areas may be explained by the results in this osmotic tolerance study that indicate *P. marinus* can use volume-regulatory mechanisms to adapt to changing external osmolality and become acclimated to extreme low osmotic conditions. Transferring infected oysters to low salinities may exacerbate the P. marinus problem by allowing acclimation of the parasites to lower salinities, thereby making them more tolerant of extremely low salinities. As a result, strains of *P. marinus* that are tolerant of a wide range of fluctuating salinities, including extremely low-salinity environments, may develop.

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PRODUCTION OF TETRAPLOID PEARL OYSTER (*PINCTADA MARTENSII* DUNKER) BY INHIBITING THE FIRST POLAR BODY IN EGGS FROM TRIPLOIDS

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ABSTRACT All previous attempts to produce viable tetraploid pearl oyster (*Pinctada martensii* Dunker) by inhibiting the first polar body and the first mitosis have failed. This study aims to test the possibility of producing viable tetraploids by the intentional process of crossing triploid females and diploid males following the inhibition of the first polar body. When 0.5mg/L CB was applied to inhibit the release of the first polar body, about 16.69% of embryos developed as tetraploids, the majority of embryos were aneuploids (65.48%); about 82% embryos developed as aneuploids in the control group (TD1), but no tetraploid embryos were found. Ploidy of embryos in the TD1 group mainly fell between 2n and 3n, but ranged from 2n to 5n in the TDCB group. During rearing period, larvae died heavily. At Day 51 post-fertilization, 2125 spat were harvested, averaging 0.033% of D-larvae cultured. Chromosome analysis revealed that 115 one-year-old pearl oysters consisted of 28.70% diploids (n = 33), 40.87% triploids (n = 47), 1.74% tetraploids (n = 2) and 28.70% aneuploids (n = 33) with 29, 30, 40, 41, and 43 chromosomes. Comparison of growth showed that aneuploids was not significantly different from diploids in both shell length and body weight ($P > 0.1$), but significantly smaller than triploids ($P < 0.05$). This study demonstrated that the production of viable tetraploid pearl oysters with eggs from triploids is possible, and certain levels of aneuploidy can be tolerated in this species.

KEY WORDS: Tetraploid, aneuploid, triploid, *Pinctada martensii* (D.)

INTRODUCTION

Artificial triploid pearl oysters, *Pinctada martensii* (D.), have been successfully obtained (Jiang et al. 1987). Because of their reduced gonadal development, triploid pearl oysters grow faster than diploids (Jiang et al. 1993), and pearls cultured in triploids are significantly bigger than pearls from diploids in pearl size, weight, and pearl layer (Lin & Jiang 1993). On the other hand, the mortality of triploids isn't different from that of diploids during the adult stage (Lin et al. 1996). All of which suggest a promising future for pearl culture by using triploid pearl oysters. Now pilot-scale testing of pearl culturing in triploids is being conducted in China. However, the method of inducing triploids by inhibiting polar bodies rarely produces 100% triploids, and treatment of induction may have deleterious effects on the survival and growth of induced triploids. If crossing tetraploids and diploids could produce all-triploids as expected, the use of tetraploids may eliminat these problems. Tetraploid is commonly induced by inhibiting the first polar body, the first mitosis division or cell fusion. However, most of previous attempts to produce viable tetraploids in several species have failed (Stephens & Downing 1988; Diter & Dufy 1990; Guo et al. 1994; Jiang et al. 1998), which has eluded researchers leading to doubt that tetraploids were inviable in shellfish. Tetraploid embryos of pearl oyster were produced with several methods, including inhibition of the first polar body, the first cleavage division with cytochalasin B (CB) or pressure, and cell fusion with PEG, but none lived to adult age (Jiang et al. 1998).

Although many attempts to induce viable tetraploids in mollusks have failed, there are a few reports of success. For example, Scarpa et al (1993) produced tetraploid mussel (*Mytilus galloprovincialis*) as an incidental product by inhibiting both the first and second polar bodies with CB treatment. Out of 29 mussels sampled at 82 days after fertilization, 5 were tetraploids (17%). Tetraploid Manila clams, *Tapes philippinarum* (Adams and Reeve), were found in offspring produced by blocking the first polar body to induce triploids (Allen et al. 1994). Guo and Allen (1994a) reported that 67% of tetraploid juveniles produced by the inhibition of the first polar body of eggs from triploid Pacific oysters (*Crassostrea gigas* Thunberg), and all-triploid Pacific oysters have been produced by mating tetraploids and diploids (Guo et al. 1996). These reports renew interests in tetraploid induction in shellfish.

This study aims to induce tetraploidy with pearl oysters by crossing triploid females and normal diploid males following the inhibition of the first polar body, and look into the possibility of this intentional process to induce tetraploid pearl oysters.

MATERIALS AND METHODS

Triploid pearl oysters, *Pinctada martensii* (D.), used in this study were produced from 2n × 2n crosses by inhibiting the first polar body with CB treatment in 1996. Ploidy was confirmed by chromosome count prior to spawning. Gametes were obtained by dissecting gonads, and were passed through a 100 μm screen to remove the large tissue debris. Fertilization was conducted at 24–25 °C. Eggs from triploid females (about 7 cm in shell length and 2.5 cm in shell width) were fertilized with sperm from normal diploid males in 0.6‰ ammonia-seawater and treated with 0.5 mg/L CB to block the release of polar body 1 (as TDCB groups). CB treatment started at 6 min after fertilization and lasted 15 min After the treatment, eggs were rinsed with 0.1% DMSO in seawater and cultured at a density of about 1/mL in filtered seawater. The remains of feed and dead larvae were removed at regular intervals to maintain water quality. The resulting spat were cultured in the sea. The first treated group (TDCB1) had one female parent; the other three groups had two triploid females respectively. The group receiving no CB treatment is as the control (TD), only the first group had a control (TD1). All groups shared one diploid male. The experiments were conducted on April. 23, 1998.

To examine the ploidy of embryos, samples of developing zygotes of 2-cell stage were taken, and treated with 0.05% colchicine

TABLE 1.

The ploidy of embryonic cells in the treated groups and the control.

Group	Diploid (%)	Triploid (%)	Tetraploid (%)	Aneuploid (%)
TDCB1	15.89	11.21	20.56	52.34
TD1	12.00	6.00	0.00	82.00
TDCB2	13.73	8.82	22.55	54.90
TDCB3	5.83	5.85	11.65	76.69
TDCB4	5.00	5.00	12.00	78.00

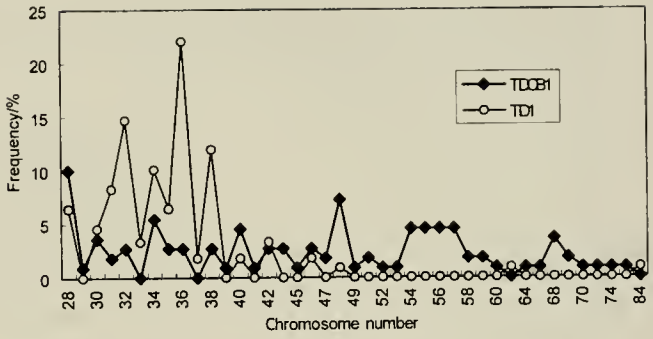

Figure 1. The distribution of chromosome number of embryonic cells in TDCB1 and TD1.

for 15 min, then fixed with Carnoy's solution (1:3 glacial acetic acid and absolute methanol). Fixatives were changed twice. Chromosomes were observed by acetic orcein stain. Briefly, drops of fixed samples were spread on a slide, stained with 1–2 drops of orcein stain (2% orcein in 50% acetic acid), and after 15–30 sec, covered with a cover glass and pressed gently. Slides were examined with a LEICA DMLS microscope; photographs were taken with black-and-white film with speed set at 100 ASA. Ploidy of embryos were determined according to $2n = 28 \pm 2$, $3n = 42 \pm 2$, $4n = 56 \pm 2$, others as aneuploids (normal diploid pearl oyster has 28 chromosomes). About 100 embryonic cells with good metaphases were counted for chromosome analysis in each group.

When pearl oysters reached 4 to 6 cm in shell length (on June 6, 1999), 230 pearl oysters were sampled. Each was numbered and measured for shell length (SL) and whole body weight (BW); a piece of gill was removed for chromosomal analysis. Gill tissues were treated with 0.05% colchicine in 50% seawater for 1h, then treated with 25% seawater (1 part seawater/ 3 parts distilled water) for 30 min, and fixed in a freshly prepared Carnoy's solution with three changes of 20 min duration. The tissue was stored overnight in fixatives in 4 °C. The next day the fixative was replaced by 50% acetic acid, gill tissue was treated for 10–30 min, then 2–3 drops of the resulting cell suspension were dropped onto a warmed slide (40–50 °C) and dried. Slides were stained with 10% Giemsa (pH6.8) for 40–60 min. Ploidy was determined by examining no less than five chromosome metaphases with the same chromosome numbers from gill cells. Individuals with 28, 42, and 56 chromosomes were classified as diploid, triploid and tetraploid respectively; any derivation from the euploid chromosome numbers was classified as aneuploid. Growth comparison between aneuploid and ploypoid was conducted by Student's *t*-test.

RESULTS

The female parent used in TDCB1 had approximately 61.92 million eggs, almost equal to normal diploids of the same size. The number of eggs obtained from seven triploid females in this study varied between 2.07 and 61.92 million. The average diameter of eggs from triploids was 57 μm, 14% larger than eggs from diploids (50 μm), which corresponded to about 50% increase in cell volume.

Chromosome examination showed that the ploidy level differed greatly among groups (Table 1). On average, there were about 16.69 ± 5.68% tetraploid embryos in the treated groups, while most of embryos were aneuploids, averaging 65.48 ± 13.75%. The percentage of aneuploids (82.00%) in TD1 was higher than that of TDCB1 (52.34%), but the percentage of polyploid (18.00%) was significantly lower than that of TDCB1 (47.66%), no tetraploids were found in the control. The distribution of chromosome numbers among embryos in TD1 and TDCB1 is showed in Figure 1. Ploidy of embryos in TD1 mainly fell between 2n and 3n, but chromosome numbers in TDCB1 ranged considerably, from 2n to 5n, and there was a peak in 4n.

The survival of the developing eggs in several stages is presented in Table 2. Due to a heavy mortality in TDCB2 and TD1, larvae were too few to be sampled for collecting data. The survival of treated groups varied greatly. At 6 days after fertilization, the mean survival rate was 24.01% of the total number of D-stage larvae cultured. At Day 22, only 2.05% of larvae survived. At Day 51, 2125 spat of 0.5cm in size were harvested. In TDCB1, 355 spat were harvested (0.0295% of D-larvae cultured), 737 spat in TDCB3 (0.0164% of D-larvae cultured), 1033 spat in TDCB4 (0.0531% of D-larvae cultured), the mean harvest rate was 0.033%.

After 1-year culture in the sea, shell length had reached 4 to 6 cm, 230 pearl oysters were sampled for size and weight, and ploidy determination. One hundred and fifteen pearl oysters had good chromosome metaphases and their ploidy was determined, the ploidy of others could not be determined due to too few metaphases. Among these 115 samples, two (1.74%) were tetraploids with 56 chromosomes, 33 (28.70%) were diploids, 47 (40.87%) were triploids, and 33 (28.70%) were aneuploids with 29, 30, 40, 41, and 43 chromosomes (Table 3). Representative

TABLE 2.

The number of D-larvae cultured and the survival at several stages in TDCB groups.

Group	Number of D-larvae cultured ($\times 10^6$)	% Survival				
		Day 6 (D-stage)	Day 12	Day 15	Day 22 (eyed stage)	Day 51 (spat)
TDCB1	1.20	30.41	24.78	3.31	0.96	0.0295
TDCB3	4.50	23.17	8.50	5.90	1.63	0.0164
TDCB4	1.95	18.45	9.99	6.31	3.57	0.0531

TABLE 3.

Individuals observed and the ploidy of adult pearl oysters from induced groups of triploid females × diploid males

	Total	Tetraploid	Diploid	Triploid	Aneuploid					
					Total	29	30	40	41	43
Number	115	2	33	47	33	10	5	2	13	3
Percentage	—	1.74	28.70	40.87	28.70	—	—	—	—	—

metaphases of ploypoids and aneuploids are presented in Figure 2.

Analysis of *t*-test showed that triploids were significantly bigger than diploids ($P < 0.05$), supporting our previous findings (Jiang et al. 1991). Aneuploids were significantly smaller than triploids in mean shell length and body weight ($P < 0.05$), but were not significantly different from diploids ($P > 0.1$) (Table 4) One tetraploid had the size of 5.38 cm in SL and 22.2g in BW, one was 5.35 cm in SL and 21g in BW. The body weight distribution of 115 samples is presented in Figure 3.

DISCUSSION AND CONCLUSIONS

Triploid shellfish are commonly assumed to be sterile due to their retarded gonadal development. Retarded gonadal development and abnormal gametogenesis have been confirmed in *Pinctada martensii* (Komaru and Wada 1990; Jiang et al. 1990) and several other species. It is interesting that some female and male triploids in mollusks can produce numeral gametes and fertilize with normal diploids, even produce offspring (Allen 1987; Allen and Downing 1990; Guo 1991; Komaru and Wada 1994; He et al.1996). In this study, 2-year-old triploid females had between 2.07 and 61.92 million eggs. Reasonable fecundity in triploid females makes it possible to produce tetraploids through this technique, but this maybe puts breeders in the unusual position of needing non-reproductive triploids for commercial culture. However, the previous research has demonstrated that the daily growth rate of triploids is obviously greater than that of diploids during the reproductive period, and there is no significant difference in most months of the non-reproductive period (Jiang et al. 1991). This result implies that a small proportion of matured triploids has no obvious effect on the advantage of faster growth. On the other hand, retarded gonadal development is not the only reason why triploids grow faster than diploids.

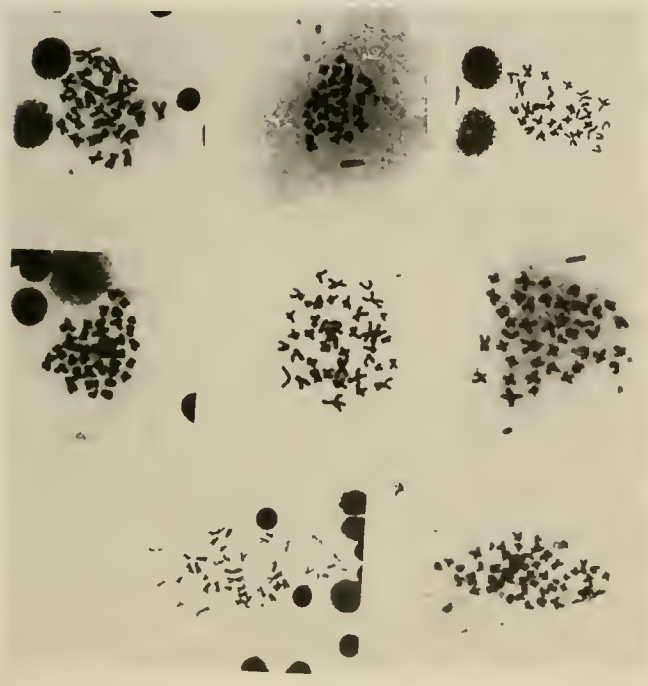

Figure 2. Representative metaphases of adult *Pinctada martensii* (D.). A: 2n = 28, B: 2n + 1 = 29, C: 2 n + 2 = 30, D: 3n − 2 = 40, E: 3n − 1 = 41, F: 3n = 42, G: 3n + 1 = 43, H: 4n = 56.

The TD cross primarily produced aneuploid embryos with chromosome number between 28 and 42, with an average ploidy of 2.5n (35 chromosomes), which agrees with previous observations (He et al. 1996). However, no larvae survived through metamorphosis in our experiments. An exception is that juvenile Japanese pearl oysters, *Pinctada fucata martensii* (a subspecies, Jiang et al. 1993), survived in TD with ploidy of 2n and 3n (Komaru and Wada 1994). This result differs from the ploidy composition (2n, 3n, and 4n) of Pacific oyster offspring in TD (Guo and Allen 1994b). In TDCB, tetraploid embryos were 16.69%, yet at adult age, only 1.74% of tetraploids survived. But, 67% tetraploid Pacific oysters were produced by this method (Guo and Allen 1994a). The percentage of adult aneuploid was 28.70%, smaller than that of early embryo stage. The ratio of diploid and triploid in adult age increased to 69.57% from 16.37% in early embryo stage. Spat harvested were about 0.033% of D-stage larvae cultured. These data suggested that most of tetraploids and aneuploids died during rearing or culturing. Guo and Allen (1994a) reported that spat of Pacific oysters were harvested from only one of three replicates, which were about 0.0738% of the developing eggs. These showed there was a heavy mortality of larvae produced by crossing triploid females and diploid males. It is concluded that lower fecundity of triploids and lower survivorship of larvae may restrict the potential of this technique for producing viable tetraploids.

Guo (1991) suggested that the inviability of induced tetraploid oysters might be due to a cell-number deficiency caused by the cleavage of eggs of normal volume with a large, tetraploid nucleus. In oysters and most other mollusks, development is mosaic. Unlike shellfish, tetraploid fish and amphibians have been obtained; their

TABLE 4.

Comparison of body size and weight between aneuploids and euploids in *Pinctada martensii* (D.).

Ploidy	Chromosome numbers	Individuals observed	Body weight (SE)/g	Shell length (SE)/cm
Diploid	28	33	23.38 (4.75)	5.07 (0.40)
Triploid	42	47	28.01 (7.80)	5.37 (0.65)
Aneuploid		33	20.62 (7.83)	4.91 (0.78)
Tetraploid	56	2	21.60 (0.85)	5.37 (0.02)

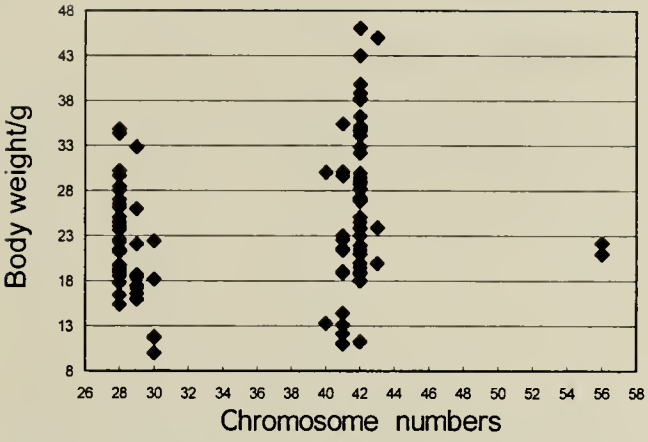

Figure 3. The body weight distribution of *Pinctada martensii* (D.) with different chromosome numbers.

development is not affected by the cell number deficiency probably because the development is regulative. The problem of cell number deficiency in tetraploid embryos might be eliminated by an increase in the egg volume. Eggs from triploids are larger than eggs of diploids, probably led to a significant reduction in cell number deficiency and therefor the survival of tetraploids. Production of viable tetraploid Pacific oysters (Guo and Allen 1994a) and pearl oysters in this study supported the cell number deficiency hypothesis. But, why are tetraploids of *Mytilus galloprovincialis* and *Tapes philippinarum* produced from eggs of diploids viable, whereas tetraploid Pacific oysters and pearl oysters produced from diploid eggs are not? Eggs of *Mytilus galloprovincialis* have a diameter of about 66–70 μm and eggs of *Tapes philippinarum* are 55–60μm, larger than the diameter of eggs from Pacific oysters (47.8 μm) and pearl oysters (50 μm). The remarkable increase in egg volume may account for it. It may reflect species-species difference in tolerance to tetraploidy. Although only two tetraploids were produced in this study, this finding indicated that tetraploidy can be tolerated in *Pinctada martensii* (D.), and demonstrated that this method of producing tetraploids is viable.

It is seen that inhibition of the first body release increased the ratio of polyploid embryos, which maybe result from a variety of segregation patterns in meiosis (Guo et al. 1992b). Que et al. (1997) reported that the pattern of chromosome segregation in meiosis was changed when CB was applied to inhibit the polar body in eggs from triploids. Four types of segregation such as tripolar segregation, united bipolar segregation, separated segregation and incomplete united bipolar segregation were observed. Similar patterns of chromosome segregation were found in pearl oysters (unpublished data). Guo and Allen (1994a) and Que et al (1997) suggested the production of tetraploids was as a result of united bipolar segregation. According to this type of segregation, the united chromosome will undergo equational division, 42 chromosomes are rejected as the first polar body, and 42 chromosomes remain in the eggs, combining with haploid sperm (14 chromosomes) producing tetraploid. In the TD cross, the majority of fertilized eggs went through two meiotic divisions and released two polar bodies, the extra set of chromosomes segregated randomly.

In addition to ploypoid pearl oysters, this process also produced many aneuploids. This study provided another evidence that certain aneuploids are viable in shellfish. The viability of aneuploid has been reported in Pacific oysters (Guo and Allen 1994a; Wang et al. 1999) and Pacific abalone (*Haliotis discus hanni*) (Fujino et al. 1990). The data showed that aneuploid pearl oysters, as a group, are not significantly different from diploids in shell length and weight. In contrast, aneuploid Pacific abalone shows no growth retardation, actually they are bigger than normal diploids (Fujino et al. 1990); aneuploid Pacific oysters (3n ± n) are larger than diploids (Wang et al. 1999), but probably because of their triploidy, not aneuploidy.

It is interesting to note one pearl oyster with 43 (3n + 1) chromosomes is the largest in body size and the second largest in body weight in this study. Guo and Allen (1994a) reported that one of the aneuploid oysters with 38 (4n - 2) chromosomes is the largest by whole body weight. These findings suggested that some aneuploids have the growth advantage and the potential application to aquaculture through breeding and selection. Certain aneuploids may also be useful in genetic manipulation. For example, trisomics and monosomics are of use of the gene transfer or gene identification. The use of aneuploid has successfully lead to the transfer of leaf rust resistance from a wild grass (*Aegilops umbellulata*) to wheat (Sears 1956).

Further research will focus on the growth and use of aneuploid pearl oysters, and on how to raise the survival rate of tetraploids.

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EVALUATION OF FIVE MICROALGAL SPECIES FOR THE GROWTH OF EARLY SPAT OF THE JAPANESE PEARL OYSTER *PINCTADA FUCATA MARTENSII*

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ABSTRACT To estimate the food value of five microalgal species, early spat of the Japanese pearl oyster, *Pinctada fucata martensii*, were fed five algal species separately; *Pavlova lutheri*, *Chaetoceros calcitrans*, *Tetraselmis tetrathele*, *Nitzschia closterium*, and *Nannochloropsis oculata*. The food value of each microalgal species was estimated from the growth rate of hinge length, dry whole spat, dry shell weight, and dry flesh weight of spat fed each microalgal diet over 19 days in the laboratory. *C. calcitrans* produced the best growth of the pearl oyster spat. *P. lutheri* and *T. tetrathele* supported moderate growth of the spat. However, growth rate of the spat fed *Nitzschia closterium* was very low and spat fed *Nannochloropsis oculata* showed negligible growth. These results indicate that *C. calcitrans* is an appropriate microalgal diet for rearing pearl oyster spat. *P. lutheri* and *T. tetrathele* are also suitable diets for rearing early spat of pearl oysters.

KEY WORDS: Pearl oyster spat, microalgal diet, growth

INTRODUCTION

Microalgal plankton is the principal food source for bivalves. There have been many studies on the nutritional value of cultured microalgae and their promotion of growth in marine bivalves larvae, spat, and juvenile (*Ostrea edulis*, Enright et al. 1986a, Walne 1963; *Crassostrea virginica*, Davis and Gullard 1958; *Saccostrea commercialis*, Nell and O'Connor 1991, O'Connor et al. 1992; *Pinctada fucata martensii*, Wada 1973, Okauchi 1990; *Crassadoma gigantea*, Whyte et al. 1990; *Ruditapes philippinarum*, Sakai and Toba 1994).

Pavlova lutheri (Droop) Green is the most popular microalgal species in Japanese bivalve culture and seed production studies (*Scapharca broughtonii*, Ohhashi and Kawamoto 1980; *Pinctada fucata martensii*, Hayashi and Seko 1986; *Ruditapes philippinarum*, Miyama and Toba 1990, Taba and Miyama 1993; *Meretrix lamarckii*, Shitomi and Kodama 1987a, Yanagida and Kodama 1988; *Pseudocardium sachalinense*, Shitomi and Kodama 1987b, Yanagida et al. 1988).

In a previous study, I reconfirmed that *Pavlova lutheri* is a suitable microalga for the growth of early spat of pearl oyster, *Pinctada fucata martensii* (Numaguchi 1999). However, there is little information that evaluates other microalgal species for the growth of pearl oyster spat. The aim of this study is to evaluate five microalgal species as diets for pearl oyster spat.

MATERIALS AND METHODS

Pearl Oyster Spat

Pearl oyster spat used were produced in the Pearl Oyster Seed Production Center of the Nagasaki Pearl Oyster Fisheries Cooperative Association. Spat were obtained approximately 3 months after fertilization in the hatchery. Average hinge length of the spat was about 3.5 mm. These spat were reared for 2 weeks in a 30-L aquarium with water temperature 26–27 °C, salinity 30–32 ppt and fed an algal diet of *Pavlova lutheri*.

Microalgae

The algal species used are shown in Table 1. Algal cultures were produced axenically in 5-L glass flasks using modified Erd–Schreiber medium: 100 mg $NaNO_3$, 20 mg $Na_2HPO_4 \cdot 12H_2O$, 50 mg $NaSiO_3$, 100 mg Nitrilotriacetic (NTA), 100 mg Tris (hydroxymethyl) aminomethane, 0.4 μg Vitamin B_{12}, 100 μg Thiamin, 1 μg Biotin, 5 mg Clewat 32 (Teikoku Kagaku Ltd, Japan; 1 g Clewat 32 contains 3.8 mg Fe, 7.7 mg Mn, 1.6 mg Zn, 0.07 mg Cu, 6.3 mg Mo, 24.7 mg B, 0.23 mg Co, and some EDTA) in 1-L of 4/5 diluted seawater. The medium was adjusted to pH 7.8 and sterilized by autoclaving (121 °C, 15 min). All species were batch cultured at 20 °C with 24-h illumination at an intensity of 5,000 lux.

Because cell size and volume differed for each of these microalgae, cell size and weight were measured for each species. Their sizes were measured using a Coulter Counter (Model ZB) and a Coulter Channelyzer-(Model C-100; Coulter Electronics Inc, USA). The range and mode of cell diameter for each algal species was estimated from the histogram of algal cell volume from Coulter Channelyzer, and the dry weight of each algal species was determined as follows. Initially, the algal cell concentration was determined using a Coulter Counter. A known volume (20–50 mL) was then filtered though a GF/C glass fiber filter (Whatman Ltd, England), which was preheated for 2 h at 500 °C to remove organic substances, to collect the algal cells. The filter was washed with 0.9% ammonium formate solution to remove salt and dried at 110 °C for 24 h. The dry cell weight was then calculated using the algal cell concentration and total weight of filtered cells. The dry weight of suspended solids in the seawater used for the experiment was also determined this way.

Experimental Design

Twenty spat were allocated to each 2-L beaker aquarium with seawater filtered with 1 μm cartridge filters. During the experi-

TABLE 1.

List of microalgal diets used for the experiment and their cellular characteristics.

Phytoplankton	Volume[1] (μm^3)	Cell Diameter[2] Range (μm)	Mode of Cell[2] Diameter (μm)	Dry Weight[3] of Cell (pg/Cell)
Haptophyceae				
Pavlova lutheri (Droop) Green	57	4.5–5.6	4.8	32.5 ± 2.6
Bacillariophyceae				
Chaetoceros calcitrans (Paulsen) Takano	56	4.5–5.6	4.8	70.3 ± 4.1
Nitzschia clasterium (HER.) W.Smith	64	4.8–7.3	5.0	30.9 ± 1.6
Prasinophyceae				
Tetraselmis tetrathele (West) Butcher	335	8.0–10.7	8.6	251 ± 10
Eustigmatophyceae				
Nannochloropsis oculata (Droop) Hibberd	9	2.3–3.5	2.6	4.9 ± 0.1

[1] Cell volume was measured by Coulter Counter and Coulter Channelyzer.
[2] Cell diameter range and mode were calculated by the equation of a spherical body from the cell volume histogram measured by the Coulter counter and Coulter Channelyzer.
[3] Values are means ± SD (n = 5).

ment, the dry weight of suspended solids in the filtered seawater was 1.54 ± 0.56 mg/L (n = 4), water temperature was 26–27 °C and salinity was 30–32 ppt.

Feeding trials, including an unfed control, were carried out over 19 days. Insufficient feeding will give false evaluations of food value of the microalgae for the growth of pearl oyster spat, so each feeding diet was supplied in excess in this experiment. Numaguchi (1999) showed that the optimal feeding concentration of *Pavlova luteri* was 2×10^4 cells/mL for maximum growth of pearl oyster spat at 2.6–3.0 mm hinge length. In this experiment, three times the concentration of *P. lutheri* (6×10^4 cells/mL) was fed to pearl oyster spat of 3.5 mm hinge length. Other algal concentrations were calculated from same packed cell volume as one of *P. lutheri*, the packed cell volume calculated to product of cell concentrations and cell volume. The feeding concentration of each algal species was set as follows; *P. lutheri* 6×10^4 cells/mL, *Chaetoceros calcitrans* 6.1×10^4 cells/mL, *Tetraselmis tetrathele* 1×10^4 cells/mL, *Nitzschia closterium* 5.4×10^4 cells/mL, and *Nannochloropsis oculata* 37.5×10^4 cells/mL.

Each algal diet was added to the relevant beaker each morning at the above concentrations. Seawater in each beaker was changed every day just before feeding to remove the remaining algae that might have negatively affected feeding. Over the rearing period, spat were observed to determine whether they were alive or dead. Spat attached to the aquarium wall were regarded as alive, and unattached spat, those with no viscera, or only a shell were regarded as dead. Dead spat were counted and removed from the aquarium.

Spat Growth Measurement

Hinge length of each spat was measured at the beginning and end of the feeding experiment using a stereoscopic microscope with a micrometer. Growth rate of spat hinge length per day was calculated as follows:

Growth rate of hinge length (μm/day) = (final average hinge length – initial average hinge length) / rearing duration

To measure the dry weight of whole spat, the spat shell, and spat flesh, ten spat were collected randomly from each aquarium at the beginning and end of the feeding experiment. Each spat was washed in 0.9% ammonium formate solution to remove salts and was wiped with paper towel. Dry whole spat weight was measured after spat were dried at 110 °C for 24 h on a platinum board. Dry shell weight was measured after drying the spat on a platinum

TABLE 2.

Growth of hinge length and mortality of pearl oyster spat.

Diet	Hinge Length (μm) Initial (0 Day)[1]	Hinge Length (μm) Final (19 Day)[1]	Growth Rate (μm/Day)	Mortality (%)
Chaetoceros calcitrans	3,405 ± 296[a] (n = 20)	8,745 ± 1,285[d] (n = 20)	281	0
Pavlova lutheri	3,443 ± 337[a] (n = 20)	6,217 ± 881[c] (n = 18)	146	10
Tetraselmis tetrathele	3,510 ± 518[a] (n = 20)	6,183 ± 921[c] (n = 18)	141	10
Nitzschia closterium	3,338 ± 373[a] (n = 20)	4,587 ± 962[b] (n = 19)	66	5
Nannochloropsis oculata	3,653 ± 330[a] (n = 20)	3,683 ± 351[a] (n = 18)	2	10
Unfed control	3,525 ± 360[a] (n = 20)	3,563 ± 346[a] (n = 15)	2	25

[1] Values are means ± SD, values within a column with different superscripts were significantly different (Duncan multiple range test, $P < 0.05$).

TABLE 3.

Dry weight gain of whole spat, shell, and flesh of pearl oyster spat.

Diet	Whole Spat[1] (μg)	Shell[1] (μg)	Flesh[1] (μg)
Initial (0 day)	1,470 ± 533	1,255 ± 452	215 ± 83
Final (19 days)			
Chaetoceros calcitrans	16,709 ± 6,250[c]	14,015 ± 4,990[c]	2,694 ± 1,302[c]
Pavlova lutheri	9,785 ± 4,180[b]	8,280 ± 3,484[b]	1,505 ± 718[c]
Tetraselmis tetrathele	7,979 ± 3,994[b]	6,852 ± 3,185[b]	1,127 ± 819[bc]
Nitzschia closterium	3,907 ± 1,447[a]	3,297 ± 1,202[a]	610 ± 275[b]
Nannochloropsis oculata	2,308 ± 674[a]	2,188 ± 548[a]	120 ± 140[a]
Unfed control	1,679 ± 410[a]	1,556 ± 389[a]	123 ± 53[a]

[1] Values are means ± SD (n = 10), values within a column with different superscripts were significantly different (Duncan multiple range test, $P < 0.05$).

board at 500 °C for 6 h in a muffle furnace to burn away the flesh. Dry whole and shell weight of each spat was weighed to the nearest 1 μg using a Micro Balance (Mettler Type M-3; Mettler Toledo, Switzerland). Dry flesh weight was calculated by subtracting dry shell weight from dry whole weight. Growth rates for the whole spat, shell, and flesh, in dry weight per day, was calculated as follows:

Growth rate of weight (μg/day) = (final average dry weight – initial average dry weight) / rearing duration

RESULTS

Table 2 shows hinge length of the spat at the beginning and end of the experiment and growth rate and mortality of the spat during the experiment. *Chaetoceros calcitrans* produced the best growth of the pearl oyster spat in this feeding experiment. Although growth rates of the spat fed *Pavlova lutheri* and *Tetraselmis tetrathele* were about half those fed *C. calcitrans*. *P. lutheri*, and *T. tetrathele*, both supported good growth rates of pearl oyster spat. Spat growth rate with *Nitzschia closterium* was poor. Moreover, there was almost no growth of pearl oyster spat fed *Nannochloropsis oculata*. There was no mortality of the spat fed *C. calcitrans* and 5–10% mortality of the spat fed *P. lutheri*, *T. tetrathele*, *Nitzschia closterium*, and *Nannochloropsis oculata*. In contrast, mortality of the unfed control was rather high (25%).

Weight gain of dry whole spat, dry shell, and dry flesh was greatest for the spat fed *Chaetoceros calcitrans*. Weight gain of the spat fed *Pavlova lutheri* and *Tetraselmis tetrathele* was moderate. Whereas, weight gain of the spat fed *Nitzschia closterium* was poor. However, spat fed *Nannochloropsis oculata* and the unfed control had very low weight gain (Table 3). Figure 1 shows the growth rate of dry spat weight, dry shell, and dry flesh of the spat fed various microalgal diets along with the unfed control. The spat fed *C. calcitrans* had the highest growth rate compared to the other microalgal species. In decreasing order, diets of *P. lutheri*, *T. tetrathele*, and *Nitzschia closterium* promoted the growth of pearl oyster spat. The spat fed *Nannochloropsis oculata* had a negative growth rate as did the unfed control.

DISCUSSION

Good growth rates of the bivalve are obtained with various algal cell because of their appropriate cell size for ingestion, their susceptibility to mechanical or enzymatic digestion by bivalves, their nutritive and biochemical composition, and their lack of toxic cell metabolite (Babinchak and Ukeles 1979).

In this experiment, *Chaetoceros calcitrans* was the superior microalgal species for maximum growth rate of pearl oyster spat. Although *Pavlova lutheri* and *Tetraselmis tetrathele* were inferior diets to *C. calcitrans*, these species supported a moderate growth rate of pearl oyster spat. These results indicate that *C. calcitrans* is an appropriate microalgal diet for rearing pearl oyster spat; whereas, *P. lutheri* and *T. tetrathele* are also suitable diets for this species. However, *Nitzschia closterium* was an unfavorable diet for the growth of pearl oyster spat. *Nannochloropsis oculata* did not promote the growth of pearl oyster spat, suggesting it is an inappropriate diet for rearing pearl oyster spat. Wada (1973) also showed that *Chlorella* sp. (now classified as *Nanochloropsis*) was a poor diet for pearl oyster larvae. Walne (1963) indicated that *Chlorella stigmatophora*, which has cell wall, is of little value as food for oyster, *Ostrea edulis*, larvae. Babinchak and Ukeles (1979) also described that the cell wall of *Chlorella autotropphica* was resistant to enzymatic breakdown by the digestive system of larvae of the oyster, *Crassostrea virginica*. *Nannochloropsis oculata* may be similarly resistant to mechanical or enzymatic digestion by pearl oyster spat.

The biochemical composition and nutritional components of microalgae differ between species (Parsons et al. 1961, Epifanio et al. 1981, Enright et al. 1986b, Whyte 1987). O'Connor et al. (1992) found that suitable dietary algal species were different for different growth stages of the same bivalve species. For the pearl oyster, Wada (1973) indicated that *P. lutheri* was a more suitable algal diet than *C. calcitrans* for larvae; however, for the spat in this experiment, *C. calcitrans* was a more suitable diet than *P. lutheri*. Furthermore, Okauchi (1990) found that *Isochrysis gracilis* was suitable algal diet for pearl oyster juveniles. These results suggest that the nutritional demands of the pearl oyster may change with its growth stage.

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Figure 1. Variation in growth rate of pearl oyster spat fed various microalgal diets. [C-cal] *Chaetoceros calcitrans*, [P-lut] *Pavlova lutheri*, [T-tet] *Tetraselmis tetrathele*, [Ni-clo] *Nitzchia closterium*, [Na-ocu] *Nannochloropsis oculata*.

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COMBINED EFFECTS OF TEMPERATURE AND ALGAL CONCENTRATION ON SURVIVAL, GROWTH AND FEEDING PHYSIOLOGY OF *PINCTADA MAXIMA* (JAMESON) SPAT

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ABSTRACT To determine a suitable culture environment to maximize growth and survival, *Pinctada maxima* spat were held at 36 combinations of temperature and algal concentration for 14 days within a flowthrough system. Survival was greatest between 23 °C and 32 °C, with 35 °C resulting in high mortalities. The optimum temperature range for *P. maxima* spat found in this study agrees well with the observed temperatures which limit the natural distribution of *P. maxima* in Australian waters. Survival of spat was highest at low algal concentrations. Growth was optimal between 26 °C and 29 °C and at 54 algal cells μL^{-1}; however, growth was still acceptable at algal concentrations as low as 12 cells μL^{-1}. The organic content increased with feeding rate and was positively correlated with specific growth rate. Spat filtration rate declined at high feeding rates, whereas grazing rate increased, with a commensurate decline in conversion efficiency. It is recommended that *P. maxima* spat be maintained within the temperature range of 26 °C to 29 °C and at algal cell densities between 12 and 54 cells μL^{-1} to maximize spat performance and minimize algal wastage.

KEY WORDS: *Pinctada maxima*

INTRODUCTION

Following high mortalities of adult silver-lip pearl oysters, *Pinctada maxima* (Pass et al. 1987), during the 1970s and early 1980s, there has been a focus on hatchery production for ongrowing and pearl production (Rose et al. 1990). Although there has been considerable work published on *P. maxima* spat production and husbandry, there has been no published investigation into the role of either temperature or food concentration on spat culture success.

Temperature is regarded as one of the most potent factors affecting growth and metabolism of marine poikilotherms (Griffiths and Griffiths 1987) and has been shown to effect many physiological processes of bivalves, such as filtration, feeding, respiration, reproduction, and growth (Bayne et al. 1976).

There is evidence that the Australian distribution of both *P. margaritifera* and *P. maxima* is limited to areas where seawater temperatures range from 18 °C to 32 °C (Hynd 1955, Pass et al. 1987). High mortalities of up to 80% of wild fished *P. maxima* in Western Australia (WA) in the late 1970s and early 1980s were attributed to reduced disease resistance during periods of low temperature (Pass et al. 1987). This effect may have been enhanced by the change in temperature (from 19 °C to 26 °C) between the collection grounds and the farms during transportation.

Rose et al. (1990) investigated the seawater temperatures of the main Western Australian fishing beds for *P. maxima* and recorded bottom temperatures of between 20 °C and 26.8 °C. Surface temperatures showed a larger range (19.8–32.3 °C).

There have been several feeding rates used for *P. maxima* spat, without any real evidence as to their suitability. Rose (1990) recommended twice-daily feedings of 55–65 cells μL^{-1}, whereas Rose and Baker (1994) fed spat a mixed algal diet at 40–285 cells μL^{-1} depending on spat size. The algal concentration dynamics in a batch-fed system will vary with tank size, stocking density, and feeding frequency.

The aim of this experiment was to quantify the effects of temperature and food availability on the growth, survival, and feeding of *P. maxima* and to determine suitable regimes for spat culture.

Materials and Methods

Experimental animals

P. maxima spat were obtained from the Darwin Hatchery Project on December 17, 1996. These spat averaged 11 ± 0.7 mg with an initial organic content of 10.9%. Mean initial shell height (dorsoventral measurement) was 4.3 mm and ranged from 3.3 to 5.2 mm.

System

The system used in this experiment was a modified and scaled-up version of that described in Mills (1997). There were three experimental blocks, each consisting of six 100-L temperature-controlled waterbaths and six elevated 100-L reservoirs. Each reservoir contained an algal suspension at one of the experimental concentrations and supplied one replicate in each waterbath through a submersible pump and 4-mm tubing manifold. The flow rate into each replicate was controlled with 2-L h^{-1} irrigation drippers. Thus, each waterbath in each block contained one replicate tray at each algal concentration, giving one replicate of each combination of temperature and food concentration per block and three replicates of each combination. Different-colored pegs were used to identify replicates of each algal concentration within a waterbath. Both the incoming air and algal suspension were preheated to the correct temperature before entering the replicates by first passing through approximately 4 m of the 4-mm supply lines coiled within the waterbath. Spat were held individually within histological cassettes, with 10 spat in each replicate tray.

Trays were supported by the rim in rectangular holes cut into a 32-mm-thick sheet of extruded polystyrene foam, which was floated within each waterbath and acted as both tray support and

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insulator. Irrigation drippers and trays were replaced at weekly intervals to prevent fouling.

Outflow from each replicate was collected from a 4-mm tube connected to the tray outlet. A 60-μm mesh feces retainer prevented contamination of the outflow sample with feces and/or pseudofeces.

Temperatures and algal concentrations

There were six temperatures used in the experiment: 20, 23, 26, 29, 32, and 35 °C. This temperature range was chosen as it encompasses the annual range experienced in Darwin Harbour (23–32 °C) and is similar to that recorded at Broome (Rose et al.1990). The ambient room temperature was maintained at 20 °C (the minimum temperature attainable in the isothermal room), and all of the waterbaths at higher temperatures were heated with 300-W glass immersion heaters. Temperatures of the replicates were checked twice daily and maintained within ±0.5 °C of the desired experimental temperature. Standard errors of experimental temperatures were generally ±0.15–0.2 °C.

The initial algal concentrations delivered from the reservoirs were 10, 20, 40, 80, and 160 cells μL^{-1}, with unfed controls exposed only to filtered seawater. All seawater was filtered to 1 μm and then passed through a carbon filter to remove possible contaminants from the intake seawater, which was drawn from a commercial shipping wharf.

The algal concentration range was chosen to encompass the optimums found for *P. fucata* (Numaguchi 1994a, Krishnan and Alagarswami 1993) and for *P. maxima* by Bellanger (1995), and also the commonly used feeding rates in commercial hatcheries (80–100 cells μL^{-1}). However, the effective algal concentration surrounding the oyster may be better represented by the concentration in the outflow (Hildreth & Crisp 1976). That the outflow algal concentration was the same as that within the replicate was confirmed by comparing the algal concentration in samples taken from several replicates at 4-h intervals, with the subsequent concentration in the outflow. Thus, the results presented are given relative to the effective (outflow) rather than initial algal concentration. The mean effective outflow concentrations were 0, 6, 12, 23, 54, and 110 cells μL^{-1}. Algal feeding reservoirs were cleaned and refilled daily with the appropriate algal suspension.

Spat were fed an algal diet of equal cell numbers of Tahitian *Isochrysis* sp. (T. Iso) and *Chaetoceros muelleri*. These species have been shown to support good growth and survival of pearl oyster spat (Taylor et al. 1997, Southgate et al. 1998). Mean algal cell dry weights were 19 and 20 pg, respectively, and were determined by the method of Epifanio (1979). Algae was cultured in 20-L carboys using f/2 medium with a 12:12 photoperiod and harvested at the late exponential stage.

Preliminary trial

A preliminary trial was conducted to determine whether there was any change in the delivered algal concentration due to cell damage, growth or sedimentation, or differences in delivery volumes due to differing friction head loss within the system. One block was run over 24 hours without animals in the system at an initial algal concentration of 100 cells μL^{-1}. Outflow volumes and initial and final algal concentrations were compared by two-way ANOVA using a significance level (α) of 0.2.

There were no significant differences in either the volumes delivered ($P = 0.56$) or the outflow concentration ($P = 0.69$). Initial and final algal concentrations were not significantly different ($P = 0.78$). Subsequent trials showed that the volume of suspension delivered by a dripper was independent of the number of drippers on the manifold line at least up to $n = 8$. This occurs as the pumps used were not positive displacement, but rather maintained a set delivery pressure and possessed a delivery capacity exceeding that of the combined number of drippers. Thus, the number or status of drippers on a manifold line had no effect on the delivery pressure (and hence output) of individual drippers.

Sampling

As it was not logistically possible to weigh and measure all of the 1080 spat and sample all of the 108 outflows during a single day, both the startup and sampling procedures were sequenced over 3 days. A full block could not be sampled on 1 day, as there was not enough floorspace for all of the outflow collection vessels; hence, a part of each block was sampled on each day. The sequence used was designed to sample one replicate of each treatment combination on each day.

At days 7 and 14, each spat was removed from the histological cassettes, washed in seawater of the appropriate temperature to remove adherent feces, and then weighed to the nearest 0.1 mg and measured to the nearest 0.1 mm (DVH). Outflow volume and collection duration were recorded, and outflow samples were preserved with Lugols iodine for later counting and calculation of filtration and grazing rates.

Growth was expressed as the daily tissue weight specific growth rate (SGR) and was calculated according to the following equation:

$$SGR = (\text{Ln final tissue weight}) - (\text{Ln initial tissue weight})/\text{time [days]}) \times 100$$

The organic content was calculated as:

$$\text{Organic content (\%)} = \text{loss on ignition/dry weight} \times 100$$

Filtration rate (FR) was calculated by the formula of Bayne et al. (1976) for flowthrough systems:

$$FR\ (\text{l h}^{-1}) = CI - CO/CO \times F$$

where CI = the initial algal concentration, CO = the final algal concentration, and F = flow rate (l h^{-1}).

This was converted to a weight-specific filtration rate by the following equation:

$$FR\ (\text{l h}^{-1}\text{g}^{-1}) = FR\ (\text{l h}^{-1})/\text{tissue weight (g)}$$

Algal grazing rate for each replicate was calculated as:

$$\text{Grazing rate (\%)} = C\ (\text{g})/\text{dry tissue wt (g)}$$

Conversion efficiency was calculated by the equation:

$$\text{Conversion efficiency (\%)} = \text{SGR/grazing rate} \times \text{organic content of algae} \times 100$$

(modified from De Silva and Anderson 1995)

Statistical analysis

All responses to temperature and algal concentration were analyzed using a two-way factorial ANOVA model. Although samples for growth, filtration, and grazing rates were taken at weekly intervals, because of the plasticity of the spat organic content the analysis was conducted only on the final values, as these responses were all calculated relative to spat tissue weight.

There was very low survival at 35 °C (1.1%), and this temperature was excluded from subsequent analysis because of the low number of surviving individuals and hence very high selection pressure on the population.

Any survival percentage data that were not normally distributed was arcsine transformed before being analyzed (Underwood 1981).

Homogeneity of variances were tested with Cochran's test with the critical value (*CV*) calculated as:

$$CV = \text{largest variance}/\ \Sigma\ \text{variance}$$

and was compared with a tabulated value with (replicates/treatment) − 1 and (treatment levels − 1) degrees of freedom.

Normality of response distributions were tested on residuals (yij-Yi) using the Shapiro-Wilk W test (Zar 1984). If variances were found to be unequal, or the data had a non-normal distribution, appropriate transformations were done. Comparison of means was only undertaken if the overall ANOVA model was significant, using the Fisher's protected least significant difference test. Relationships between measured responses and culture conditions were examined using regression analysis. A P value <0.05 was considered significant for all statistical analysis.

Results

Survival

Both temperature ($P < 0.0001$) and algal concentration ($P = 0.03$) affected spat survival, with temperature being a much stronger influence than algal concentration. There was no significant interaction ($P = 0.16$). Within the naturally occurring temperature range for Darwin Harbour (23–32 °C), there was no effect of temperature on mortality rates, and survival was greater than 90% (Fig. 1). At 35 °C mortality was almost complete (98.9%), and at 20 °C survival was significantly lower than at 23 °C, 26 °C, 29 °C, and 32 °C.

Spat at the lowest algal concentration of 6 cells μL^{-1} showed the highest survival, which was significantly higher than those at 23 and 110 cells μL^{-1} and unfed spat (Fig. 2). It is notable that the only survival at 35 °C was at the lower algal concentrations (6 and 23 cells μL^{-1}).

Growth

Tissue SGR responded significantly to both temperature and algal concentration ($P = 0.0008$ and $P < 0.0001$, respectively), but there was no significant interaction ($P = 0.73$). Growth increased with increasing temperature up to 29 °C and then declined from 29 °C–32 °C (Fig. 3). The decrease in growth at 32 °C indicates that this is approaching the upper temperature limit for the species, as confirmed by the very low survival at 35 °C. Growth at 29 °C was more than twice that at 20 °C, and growth at 32 °C was similar to that at 23 °C. Tissue weights of unfed spat declined, indicating that there was no significant nutritional value in the filtered seawater. In fed spat, growth increased progressively with increasing algal concentration up to 54 cells μL^{-1}, after which there was a slight but nonsignificant decline (Fig. 4). Growth at 54 cells μL^{-1} was approximately 50% greater than that at 6 cells μL^{-1}. This illustrates that *P. maxima* spat are capable of moderate growth even at very low algal concentrations.

Organic content

Increases in algal concentration were reflected in significant increases in spat organic content ($P = 0.0002$), from 9.4% in unfed spat to >13% at the highest concentrations (Fig. 5). The organic content of spat cultured at 6 and 12 cells μL^{-1} was not significantly different from the initial value of 10.9%. Temperature had no significant effect on spat organic content ($P = 0.8$), nor was there any significant interaction between temperature and algal concentration ($P = 0.8$). There was a positive correlation between the SGR of spat and their organic content ($r^2 = 0.51$, P

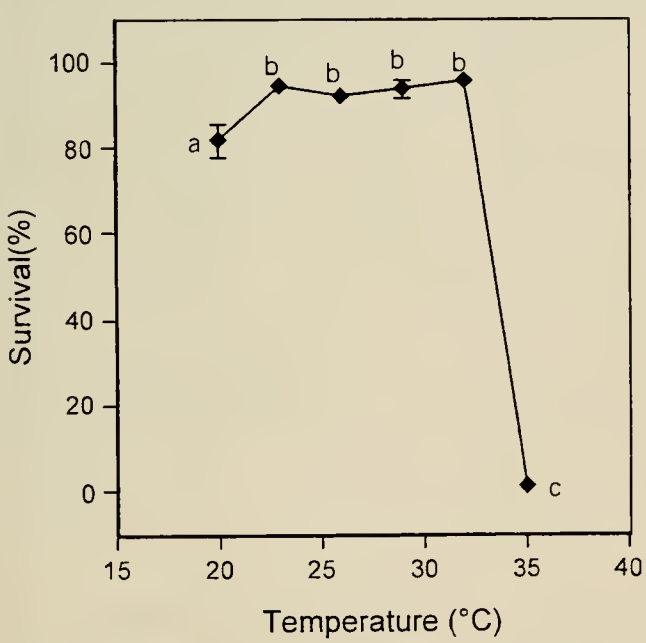

Figure 1. Survival of *P. maxima* spat after 14 days' culture at various temperatures. Figures show means ± standard error. Means with similar subscripts are not significantly different ($P > 0.05$).

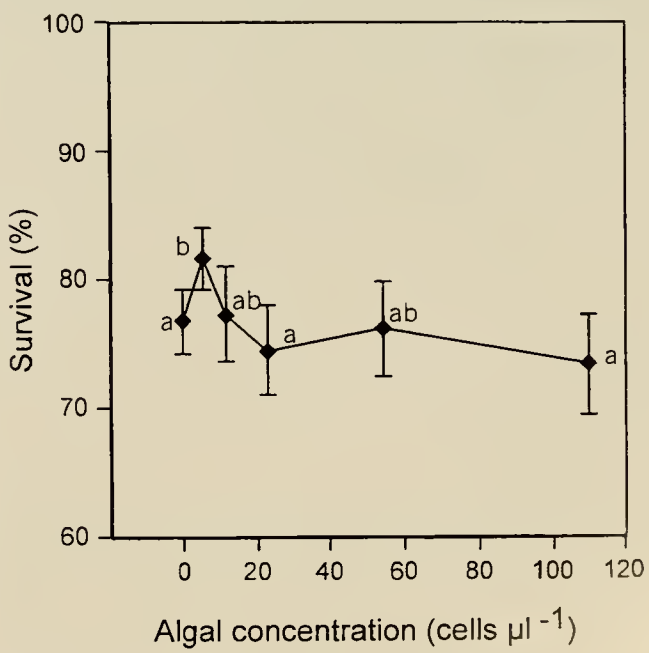

Figure 2. Survival of *P. maxima* spat after 14 days culture at various algal concentrations. Figures show means ± standard error. Means with similar subscripts are not significantly different ($P > 0.05$).

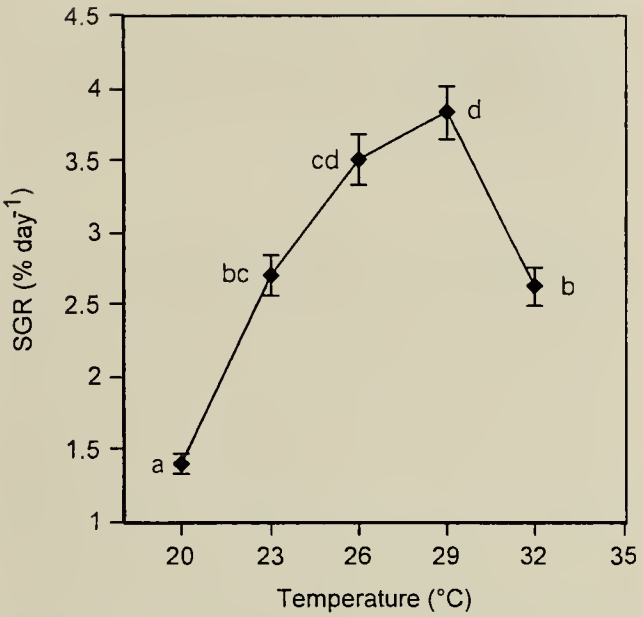

Figure 3. SGR of *P. maxima* spat at various temperatures. Figures show means ± standard error. Means with similar subscripts are not significantly different ($P > 0.05$).

< 0.0001), with faster-growing spat having a higher organic content.

Feeding

The two different algal species comprising the diet were counted separately in outflow samples obtained during the first week. There was no preferential selection by the spat for either of the species at any concentration or temperature, and the ratio of T. Iso to *C. muelleri* in the outflow was not significantly different from 1. Henceforth, for the calculation of feeding rates it was assumed that there was no selection for either species by the spat.

The weight-specific filtration rate increased with moderate increases in algal concentration up to 23 cells μL^{-1}, before declining significantly at 54 and 110 cells μL^{-1} (Fig. 6). Filtration rate was highest at 20 °C (54 L $h^{-1}g^{-1}$) and declined significantly with increasing temperature to 17.3 L $h^{-1}g^{-1}$ at 32 °C (Fig. 7). This is an inverse response to that shown in most bivalve studies, in which

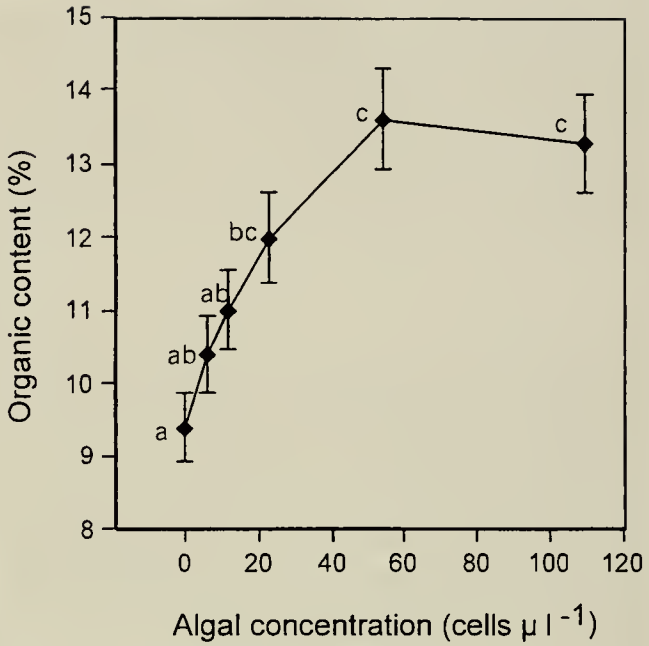

Figure 5. Organic content (% of dry weight) of *P. maxima* spat after 14 days at various algal concentrations. Figures show means ± standard error. Means with similar subscripts are not significantly different ($P > 0.05$).

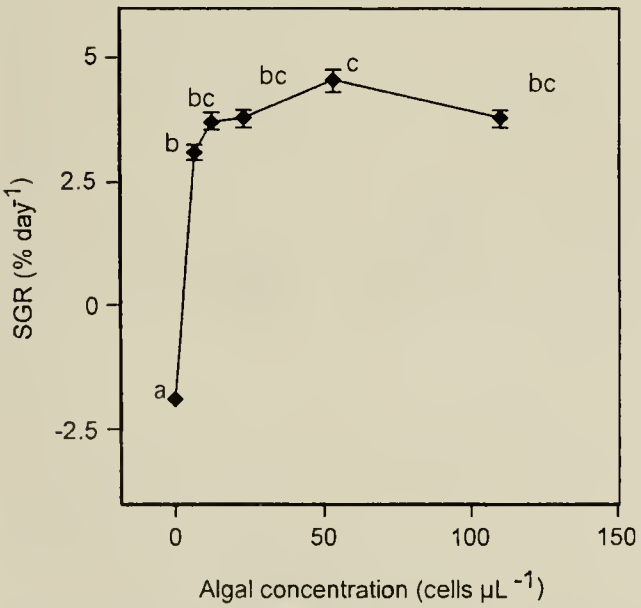

Figure 4. SGR of *P. maxima* spat after 14 days at various algal concentrations. Figures show means ± standard error. Means with similar subscripts are not significantly different ($P > 0.05$).

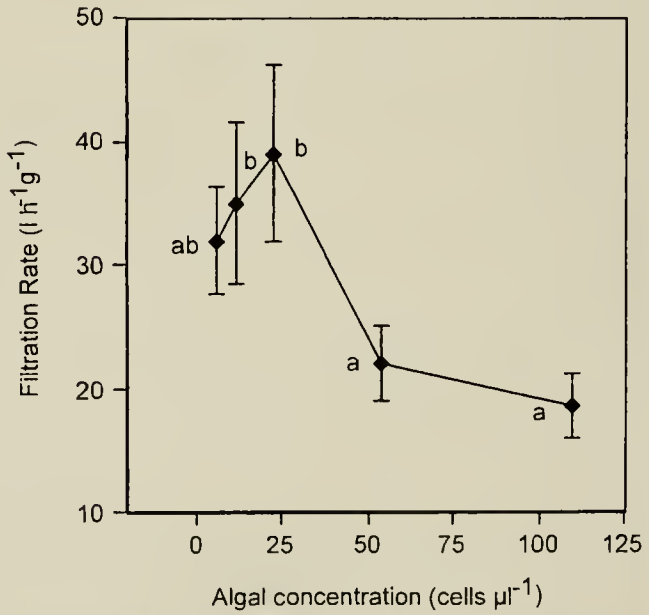

Figure 6. Filtration rates of *P. maxima* spat at various algal concentrations. Figures show means ± standard error. Means with similar subscripts are not significantly different ($P > 0.05$).

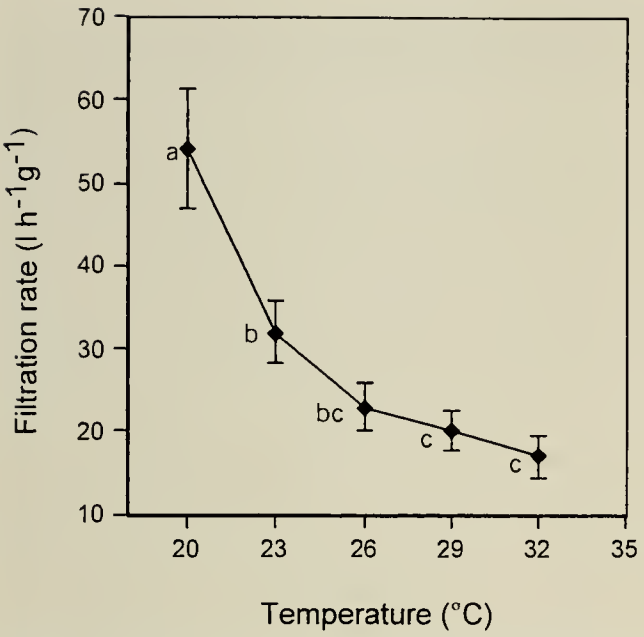

Figure 7. Filtration rate of *P. maxima* spat at different temperatures. Figures show means ± standard error. Means with similar subscripts are not significantly different ($P > 0.05$).

filtration rate generally increases with increasing temperature up to a maximum, with a subsequent decline.

The grazing rate (G) increased linearly with increasing algal concentration from approximately 15% at 6 cells μL^{-1} to 136% at 110 cells μL^{-1} following the equation:

$$G\% = 0.74 \times \text{algal concentration} + 18.2 \ (r^2 = 0.96) \text{ (Fig. 8)}$$

With the increase in grazing rate, there was a corresponding decline in the gross conversion efficiency from approximately 38% at 6 cells μL^{-1} to 5% at 110 cells μL^{-1} (Fig. 8). As the production of pseudofeces was not quantified, the gross conversion efficiency refers to growth from algae grazed, rather than ingested. The logarithmic decline in conversion efficiency (CE) can be described by the equation:

$$CE = [128.4 \times \text{algal concentration (cells } \mu L^{-1})]^{-0.745}$$

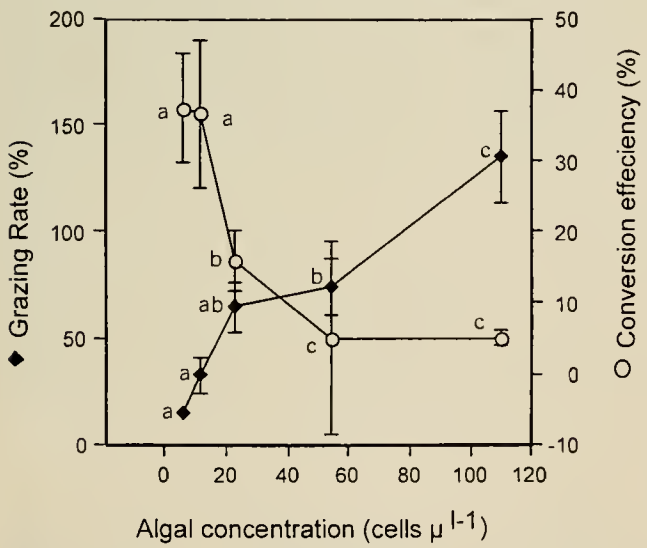

Figure 8. Grazing rate and conversion efficiency of *P. maxima* spat at different algal concentrations. Figures show means ± standard error. Means with similar subscripts are not significantly different ($P > 0.05$).

As the grazing rate increased greatly in response to increasing algal concentration without a commensurate increase in growth rate, it seems likely that the majority of the algae grazed were rejected as pseudofeces. Neither the grazing rate nor conversion efficiency was affected by temperature, nor was there any significant interaction of the two factors.

Discussion

Survival

The pattern of survival exhibited in this experiment is consistent with the observations of Pass et al. (1987), who concluded that the natural distribution of *P. maxima* was limited to areas with a seawater temperature range of 18–32 °C. Although it was not possible to examine the effects of temperatures below 20 °C, it is apparent from the significantly lower survival at 20 °C that the spat were approaching their lower tolerance limit. The very low survival at 35 °C indicates that this is above the upper tolerance limit, as foreshadowed by the reduced growth at 32 °C. The reduction in survival at 20 °C, and the very low survival at 35 °C, reflects the results of Doroudi et al. (1999) for *P. margaritifera* larvae, in which there was no development at either 20 °C or 35 °C. The optimum range for *P. margaritifera* larvae was found to be between 26 °C and 29 °C, which is slightly narrower than that found for *P. maxima* spat in the present study (23–29 °C). Numaguchi and Tanaka (1986) investigated the effects of temperatures from 7.5–35 °C on *P. fucata* and concluded that the lower and upper tolerance limits were 15 °C and 32 °C, respectively, with the optimum range being from 17.5 °C to 28 °C.

Temperatures on both the natural pearl oyster beds and pearl farms in WA would occasionally be high or low enough to be deleterious to *P. maxima* spat, as they generally range from 20 °C to 32 °C (Rose et al. 1990). In the Northern Territory (NT), summer inshore water temperatures are generally 31–32 °C from December to April (Padovan 1997) and may be approaching stressful temperatures. Wada (1953) observed that the temperature of the main deepwater commercial oyster grounds in the N.T. was the same throughout the water column and averaged 29 °C during the summer. The lower temperatures offshore may be more conducive to growth and reproduction than warmer inshore waters.

As surface seawater temperatures reach higher levels than bottom waters, pearl oysters hung from longlines (typically 1–3 m deep) may experience temperatures greater than 32 °C, especially in sites farther to the north such as the Kimberly region in northern WA, and the NT, and in calm sheltered bays. These temperatures may be at or near the tolerance limit of the species, and although there does not seem to be any direct mortality associated with them, there could be significant sublethal effects such as reduced growth, reproductive output, pearl quality, and resistance to stressors such as cleaning and handling. This may account for the lower-quality spawnings and gametes produced by oysters from farm longlines compared with those from the offshore fishing grounds (Rose et al. 1990), although this may be partly attributable to the frequent cleaning and handling of farm oysters.

High water temperatures may be more critical in NT hatcheries, as during the summer air temperatures commonly reach 34 °C, and this may be reflected in the temperature of the rearing tanks. Cur-

rently, the industry addresses this by shading of the seawater supply and rearing tanks; however, temperatures may still reach stressful levels. Stressed larvae and spat may be more susceptible to disease and suboptimal water quality. Algal cultures used to feed larvae and spat are grown at temperate water temperatures (20–24 °C), and problems may occur as a result of the abrupt increase in temperature experienced by the algae when it is transferred from the algal culture system to the spat culture tanks. Minaur (1969) noted that *P. lutheri* became moribund at temperatures above 30 °C and attributed this as a major problem in attempts to rear *P. maxima* larvae and spat.

Numaguchi (1994b) considered that an increase in the mortality rates of farm-held *P. fucata* was due to sustained elevated temperatures of greater than 28 °C. This may have been related to temperature stress combined with reduced food intake and higher metabolic costs, as the same author demonstrated that filtration rate declines dramatically at temperatures above 28 °C (Numaguchi 1994c), whereas catabolic losses increase at higher temperatures (Numaguchi 1995). There appears to be a similar process in *P. maxima*. Assuming that the organic content of the spat at day 7 was the same as that at the beginning of the experiment, then at 35 °C the mean filtration rate over all algal concentrations at 35 °C was 9.6 L $h^{-1}g^{-1}$ compared with >30 L $h^{-1}g^{-1}$ at temperatures from 20–32 °C. Unfed spat showed greater tissue weight loss with increasing temperature. This strongly suggests that if the experiment had been extended, there would have been large mortalities in unfed spat, particularly at the higher temperatures. The combination of these two factors indicate that at 35 °C there is reduced feeding and increased metabolic costs, leading to negative growth and increased mortality.

Algal concentration had a small but significant effect on spat mortality, with the survival rates at 6 cells μL^{-1} significantly greater than those of unfed spat and those at 23 and 110 cells μL^{-1}. The only surviving spat at 35 °C were unfed, and at the lower fed algal concentration. This is probably a result of the stimulatory response in filtration rate at higher algal concentrations (Fig. 6), resulting in an increase in metabolic rate and energetic costs, and consequently a more rapid loss of body tissue and death.

Growth

As previously found by Mills (1997), there was no significant relationship between the initial weight of spat and the subsequent SGR within the spat size range used ($r^2 = -0.11$, $P = 0.08$).

Increasing growth with increasing temperature up to an asymptotic point, followed by a rapid decline, is a common pattern for bivalves (Bayne et al. 1976). A similar pattern was shown by a temperate strain, *P. fucata* (Numaguchi and Tanaka 1986), exposed to temperatures ranging from 7.5 °C to 35 °C.

The relationship between preasymptote temperature and growth of *P. maxima* spat can be described by the second-order polynomial equation:

$$\text{Growth (SGR \% day}^{-1}) = -19.85 + 1.6 \text{ temperature} - 0.027 \text{ temperature}^2 \ (r^2 = 0.98)$$

From this equation, the calculated temperature of zero growth is 17.7 °C, which agrees well with the estimate of the lower temperature limiting the distribution of *P. maxima* by Pass et al. (1987) of 18 °C.

Growth relative to algal concentration showed a pattern similar to that obtained by Numaguchi (1994a) for *P. fucata* spat, in which growth increased rapidly up to a concentration of 20 cells μL^{-1}, with no advantage of further increases in concentration. In this study, growth increased rapidly up to 12 cells μL^{-1}, with further increases in concentration producing slightly higher growth. This lower threshold value for *P. maxima* may reflect the very high filtration rates attainable in this species. Yukihira et al. (1998b) calculated that the algal concentration for maximum scope for growth (SFG) of adult *P. maxima* was 20–30 cells μL^{-1}. Above this concentration the calculated SFG declined and was negative above 90 cells μL^{-1}. The decline was primarily due to a large reduction in the absorbed energy as a result of a decrease in absorption efficiency. Similarly, the SFG of *P. maxima* spat calculated by Bellanger (1995) predicted that growth would decline at T. Iso concentrations greater than 17 cells μL^{-1}. Although the results of the present study indicate that low algal concentrations may still promote good growth, there is no evidence that higher algal concentrations are detrimental. Bellanger (1995) could not separate pseudofeces from true feces, and consequently the absorption efficiency was underestimated. It is possible that spat may have different energetic characteristics than adults. Alternatively, there may be an acclimation to higher algal concentrations over time, which cannot be compensated for in short-term studies.

Preasymptotic growth at various algal concentrations can be described by the equation:

$$\text{Growth (SGR\% day}^{-1}) = 2.921 + 0.05 \text{ algal concentration (cells } \mu L^{-1}) - 3.795^{-4} \text{ algal concentration (cells } \mu L^{-1})^2 \ (r^2 = 0.93)$$

This equation predicts a maintenance ration (where SGR = 0) of 1.45 cells μL^{-1}. This value is substantially lower than that of Bellanger (1995), where the SFG was calculated to be 0 at 7.6 cells μL^{-1}. Given that growth was still quite high at 6 cells μL^{-1} in this experiment, the estimate obtained in this study would appear to be a more accurate estimate of the maintenance concentration. Yukihira et al. (1998a) calculated that the SFGs for *P. maxima* and *P. margaritifera* were very high even when exposed to very low algal concentrations (5 cells μL^{-1}). Hayashi and Seko (1986) monitored chlorophyll *a* levels and growth of *P. fucata* on pearl farms in Japan and concluded that maintenance requirements were met by algal concentrations that result in chlorophyll *a* levels of 3 $\mu g\ L^{-1}$, whereas levels of 4–5 $\mu g\ L^{-1}$ were required for good growth and reproductive development. This was equivalent to 6 and 10 cells μL^{-1} of *P. lutheri*, respectively. *P. fucata* appears to be adapted to more eutrophic conditions than *P. maxima*, as chlorophyll *a* levels in Darwin Harbour are generally from 0.5 to 3 $\mu g\ L^{-1}$ (Padovan 1997) and similarly low levels occur in the main fishing grounds off Broome (Rose et al. 1990). Mean chlorophyll *a* levels recorded at the Broome fishing grounds were from 0.3 to 0.9 $\mu g\ L^{-1}$ (Rose et al. 1990), equivalent to approximately 0.6–1.8 cells μL^{-1}. Thus, the calculated maintenance ration in this experiment agrees well with observed food levels in the field. The ability to thrive in conditions of very low food concentrations is due to the ability to process very large volumes of water (Yukihira et al. 1998a). As growth rates increased by 50% from 6 to 54 cells μL^{-1}, it may be that growth of oysters in the field is commonly food limited.

Organic content

Organic content is rarely determined in bivalve studies, and there are few references to pearl oyster spat. Given that spat organic content increased with both algal concentration and SGR, and that SGR increased commensurately with algal concentration, it is possible that the increase in organic content is related to the SGR rather than the algal concentration *per se*. This would agree

with the results of Taylor et al. (1997), who found that the organic content of *P. maxima* spat increased with higher SGR despite a reduced weight-specific ration fed to the fastest-growing spat.

Feeding

A limitation of the experimental method utilizing histological cassettes is that it is impossible to collect biodeposits; thus, the estimation of ingestion, absorption, and conversion efficiencies cannot be carried out. The filtration rates obtained in this experiment are very high compared with those of the previous experiment and other published filtration rates. This is probably due to the small size of the spat used, as the weight-specific filtration rate generally declines with increasing size according to the equation:

$$FR\ (\text{L h}^{-1}) = \text{aW}^{\text{b}}\ \text{(Bayne et al. 1976).}$$

Thompson and Bayne (1972) found that the weight exponent for mussels less than 1 g dry weight was higher than that of larger mussels. Thus, the very high filtration rates found in this study may reflect the very small spat used. Yukihira et al. (1998a) demonstrated that smaller *P. maxima* and *P. margaritifera* spat had a considerably higher filtration rate than larger animals. Using the equation developed by Yukihira et al. (1998a), $CR = 10.73\ W^{0.617}$, the predicted filtration rate for the mean final spat ash-free dry weight used in this trial (0.0023 g) would be 0.115 L h^{-1} compared with a measured value of 0.09 L h^{-1} .

Yukihira et al. (1998a) found that the filtration rates obtained for *P. maxima* and *P. margaritifera* were among the highest recorded for any bivalve species. A similar result was found for *P. margaritifera* by Pouvreau et al. (1999).

Reduction in filtration rate is a common response to increasing algal concentration (Bayne et al. 1976). The trends found in this study are similar to the findings of Bellanger (1995), in which algal concentrations above 17 cells μL^{-1} resulted in a decrease in the weight-specific filtration rate. In both cases, filtration rates initially increased with moderate increases in algal concentration and then declined at higher algal concentrations.

Reduction in filtration rate with increasing temperature is contrary to results from other studies on pearl oysters. Numaguchi (1994c) found that the filtration rate of 2-year-old *P. fucata* (5.7–6.1 cm shell height) increased with increasing temperature up to the tolerance limit before sharply declining. A similar pattern was shown for *P. fucata* spat (Numaguchi 1994a). The unusual results in this study are probably an artifact of the differences in size of the spat at the various temperatures, and a high rate exponent. Mean final dry tissue weight at 20 °C was 7 ± 1.5 mg and increased commensurately with temperature up to 21 ± 1.7 mg at 32 °C. Filtration rates of the largest spat (17 L $h^{-1}g^{-1}$ at 32 °C) approached those obtained by Mills (1997) of 7.3 L $h^{-1}g^{-1}$ and those of Bellanger (1995) (11.9 L $h^{-1}g^{-1}$). To eliminate any potential effects of different-sized spat, a short-term experiment would have to be conducted with similar-sized spat at all temperatures.

The lack of a temperature effect on grazing rate may also be an artifact of the variations in spat size at the different experimental temperatures, as the increase in filtration rate by smaller spat would have masked the increase in grazing rate with higher temperatures.

The increase in grazing rate with increasing algal concentration reflects the relatively low corresponding decrease in filtration rate. As growth did not increase proportionally, the extra algae grazed at higher algal concentrations was probably rejected as pseudofeces. This was consistent with observations that pseudofeces were produced at initial algal concentrations above 20 cells μL^{-1}. A similar observation was made by Bellanger (1995) at algal concentrations greater than 22 cells μL^{-1}. This increased rejection as algal concentration increases is reflected in the decrease in conversion efficiency from approximately 37% at 6 and 12 cells μL^{-1} to approximately 5% at 54 and 110 cells μL^{-1}.

Both the grazing and growth rates in this study are substantially higher than those recorded by Mills (1997), suggesting that growth is heavily dependent on food intake. This is consistent with the higher growth at higher algal concentrations.

In the present study the growth rate at 6 cells μL^{-1} was still quite high, although Bellanger (1995) predicted it to be negative. It is likely that the metabolic costs in that study were overestimated, leading to erroneous conclusions as to the predicted growth at various algal concentrations.

On the basis of the results of this study, *P. maxima* spat should be maintained at temperatures between 26 °C and 29 °C, and algal concentrations between 12 and 54 cells μL^{-1}. Within these culture parameters, spat growth and survival will be optimal, and the efficient utilization of algal cultures will be maximized.

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INFLUENCE ON UPTAKE, DISTRIBUTION AND ELIMINATION OF *SALMONELLA TYPHIMURIUM* IN THE BLUE MUSSEL, *MYTILUS EDULIS*, BY THE CELL SURFACE PROPERTIES OF THE BACTERIA

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ABSTRACT This study was carried out to investigate whether the cell surface charge of *Salmonella typhimurium* could influence the kinetics of uptake, distribution, and elimination in the blue mussel, *Mytilus edulis*. The bacteria (1 μm) were labeled with $^{99}Tc^{m}$ in the presence of stannous fluoride. Two different concentrations of stannous fluoride were used to produce differences in the cell surface charges of the bacteria. A set of mussels in the investigation were also given ^{113}Sn-labeled microspheres (15 μm) together with bacteria to compare the impact between particle size and cell surface properties on the distribution kinetics. The distribution of radiolabeled particles in the mussel was followed and analyzed with a computer-aided gamma camera that can detect two isotopes simultaneously. Finally, the mussels were dissected and the radioactivity in the fractions was measured with a well-shielded NaI(Tl) detector. The reduced cell surface charge of *S. typhimurium* enhanced the preingestive selection on the gills or labial palps as well as the postingestive selection in the digestive glands in such a way that it became similar to the microspheres, despite the size differences. The uptake of the bacteria labeled in the presence of less stannous fluoride was significantly lower. However, the subsequent absorption of these bacteria in the digestive gland was greater, because the recovery of radioactivity outside the digestive tract was higher than for the more manipulated bacteria and the microspheres. Likewise, the elimination of the more manipulated bacteria was similar to that of the microspheres and significantly higher than that of the less affected bacteria. It is concluded that the cell surface properties of bacteria, possibly the charge, influence the uptake, distribution, and elimination in *M. edulis* and that this factor could have the same influence as size on the uptake capacity.

KEY WORDS: *Mytilus edulis*, bivalves, molluscs, *Salmonella typhimurium*, gamma camera, $^{99}Tc^{m}$-labeled bacteria, surface, uptake, preingestive selection, postingestive selection

INTRODUCTION

Because of their efficient filter-feeding mechanism, bivalves are capable of accumulating large numbers of microorganisms from the surrounding water.

Problems related to microbes in terms of pathogenic bacteria and viruses in bivalves can be a major deterrent when developing a sustainable plan for utilization of coastal resources. The planktonic bacterium *Vibrio parahaemolyticus*, as well as bacteria associated with fecal pollution, such as *Salmonella*, *Shigella*, and *Clostridium spp.*, have caused numerous outbreaks of gastroenteritis in connection with consumption of seafood (Matches and Abeyta 1983, Rodrick and Schneider 1991, Wilson and Moore 1996). In addition, viruses, such as the small, naked viruses (e.g. Calici and Norwalk viruses), hepatitis A, and enterovirus, are considered as health hazards in utilizing bivalves for food (Sindermann 1990, Enriques et al. 1992, Cliver 1997). To improve risk assessment and develop satisfactory control methods in respect to public health, the basic knowledge about the regulating mechanisms for uptake, distribution, and elimination of microbes in bivalves has to be improved.

A study on filtration capacity of particles in *Mytilus edulis* (Møhlenberg and Riisgård 1978) showed a marked decline in the uptake of particles smaller than 7 μm, which fell to 20% at 1 μm. Also, it has been shown by Allison et al. (1998) that particles (3–40 μm) enriched in metals were rejected in *M. edulis*, and microalgal metabolites have been proved to influence mussel feeding behavior (Ward & Targett 1989). This indicates that the lamellibranch bivalves have some ability of preingestive selection, presumably on the gills or the labial palps, which is not only related to size but also to other particle characteristics. In addition, the possibility of postingestive selection in the digestive tract has been suggested (Shumway et al. 1985, Smith & MacDonald 1997). Digestion in invertebrates includes extracellular and intracellular digestion processes. The extracellular digestion is a fast process that dominates during intestinal digestion. It results in low absorption efficiency and poorly digested "intestinal" feces. The intracellular digestion is a slow process in the diverticular folds of the stomach. The epithelial cells of the folds phagocytose and digest small particles with high efficiency resulting in good absorption and well-digested "glandular feces" (Morton 1973, Decho & Luoma 1991). The hemocytes of the bivalves are also functioning in nutrient digestion and transport as well as in internal defense (Cheng 1984). They contain numerous lysosomes capable of releasing hydrolytic enzymes and reactive oxygen radicals (Winston et al. 1991). Birkbeck and McHenery (1982) showed that bacteria such as *Micrococcus roseus* and *Staphylococcus aureus*, resistant to the lysozyme of *M. edulis*, were rejected intact, whereas *Escherichia coli*, *M. luteus* and *Bacillus cereus*, which were sensitive to lysozyme, were killed after ingestion. Rögener & Uhlenbruck (1984) found that invertebrates recognized and bound so-called

heterophilic antigens or ubiquitous chemical structures such as lipopolysaccharides and zymosan, which are often present on the surface of microorganisms. This indicates that recognition of cell surface characteristics of the prey might be a regulating mechanism for selection in the digestive gland.

We hypothesize that not only size but also cell surface properties of particles might influence the uptake and the subsequent distribution and elimination of microorganisms in *M. edulis*. To test this, a gamma camera technique was used for *in vivo* recording of blue mussels with respect to uptake and elimination of radiolabeled *Salmonella typhimurium* (ca. 1 μm) and microspheres (15 μm). After the gamma camera experiment, the radioactivity in dissected fractions of the mussel tissue was measured with well-shielded NaI(Tl) detector to follow the distribution into different organs. The labeling procedure for *S. typhimurium* has been described in the previous study by Hernroth et al. (2000). Bacteria with different cell surface charges were obtained by using different concentrations of stannous fluoride.

MATERIALS AND METHODS

Bacterial Strain and Growth Conditions

S. typhimurium 395 MR10, a nonvirulent, chemotype-Rd mutant (Edebo et al. 1980) was grown for 16 h in glucose broth (Lindberg et al. 1970) (pH 7.0–7.2) at 37 °C on a rotary shaker (200 rpm). The bacterial suspension was washed three times by centrifugation (2000 rpm, 10 min, 4 °C) in 3 mL 0.9% NaCl to remove the culture medium. The pellet was resuspended in 2 mL 0.9% NaCl (2.5×10^9 mL^{-1}).

Radiolabeling and Chemical Modification of Bacteria

Stannous fluoride (SnF_2) was used to reduce $^{99}Tc^m$ to facilitate the labeling of the isotope (Perin et al. 1997). It also binds to protein structures intracellularly as well as at the cell surface (Rhodes 1991). It has been shown in a previous study by Hernroth et al. (2000) that stannous fluoride can chemically modify the cell surface charge of *S. typhimurium*. The bacteria showed differences in electrophoretic mobility when 80 μg SnF_2 and 800 μg SnF_2, respectively, were used in the labeling procedure. The mobility for bacteria treated with 80 μg SnF_2 (5.4×10^{-9} m$^2 \times$ V^{-1}s^{-1}) was not significantly different from untreated *S. typhimurium* (4.7×10^{-9} m$^2 \times$ V^{-1}s^{-1}), whereas the mobility for bacteria treated with 800 SnF_2 was significantly reduced (2.3×10^{-9} m$^2 \times$ V^{-1}s^{-1}). The same treatment to label bacteria with different cell surface properties was used for this study.

One milliliter of bacteria suspension (2.5×10^9 mL^{-1}) was mixed with 2 mL of 37 °C 0.9% NaCl containing 80 or 800 μg SnF_2 and then incubated with approximately 50 MBq $^{99}Tc^m$-pertechnetate for 20 min at 37 °C on a rotary shaker (200 rpm). After incubation, the bacteria were centrifuged and washed three times. Ascorbic acid (0.25 mg mL^{-1}) was added to the washing solution to prevent reoxidation of the reduced $^{99}Tc^m$. The bacteria were resuspended in 1 mL 0.9% NaCl.

To control the cell size and shape of *S. typhimurium* and observe possible formation of aggregates, the batches of labeled bacteria were inspected in a microscope (12.5 × 100× magnification) before feeding took place. As references, unlabeled *S. typhimurium* bacteria were used.

Viability of ^{99m}Tc-Labeled Bacteria

The viability of the bacteria was checked using a fluorescence assay (LIVE/DEAD *Bac*Light™ Bacterial Viability Kit, Molecular Probes, The Netherlands). Triplicates of $^{99}Tc^m$:80 and $^{99}Tc^m$:800 were compared with unlabeled bacteria from the same culture, using epifluorescence microscopy (Zeiss Axioscop, Exciterfilter BP450-490, Dichroic reflector 510, and Barrier filter LP5159, Zeiss, Germany). The suspension of bacteria was diluted to $10^6 \times$ mL^{-1} in sterile filtered (Schleicher & Schuell, Keene, NH, FP 030/3) seawater (32 PSU) and incubated with the fluorescence probe for 15 min at 12 °C. The intact plasma membranes of live bacteria give green fluorescence, whereas compromised membranes of dead ones give red fluorescence (Haugland 1996). The viable bacteria were calculated as part of 100 cells.

Mussel Experiment

The experiment was carried out during April and May 1998. Blue mussels, *M. edulis*, were collected at 1 m depth in the Åby Fjord, on the west coast of Sweden (tidal amplitude 20 cm). The salinity, when sampling, was 28 PSU, and the temperature was 6 °C. Mean shell length of the 32 mussels was 7.6 ± 0.8 cm, shell width was 3.4 ± 0.7 cm, and flesh wet weight was 11.6 ± 3.5 g. The mussels were cleaned from epiphytes and stored in running seawater (32 PSU and 8 °C). Two days before the experiment started, mussels were placed individually in hanging baskets in filtered (Millipore, 0.3 μm) seawater (32 PSU, 12 °C) for adaptation to the experimental setup, in which it was necessary to mix the water by magnetic stirring. The water (eight mussels in 15 L) was exchanged daily. It was continuously oxygenated, and the mussels were fed the nanoflagellate *Isochrysis galbana*. One hour before the start of the experiment, each basket with one mussel was transferred to a beaker with 700 mL filtered (Millipore, 0.3 m) and oxygenated seawater (32 PSU, 12 °C). The beaker was placed on the magnetic stirrer in front of the gamma camera. Ten minutes before addition of bacteria, the mussel was given 1 mL of a suspension of 10^6 mL^{-1} of *I. galbana* as a trigger for filtration, and the activity was visually confirmed. Thereafter, the radiolabeled microspheres and/or bacteria were added to the beaker. The final concentration of bacteria was approximately 3 10^6 mL^{-1}, and approximately 32.4 MBq of $^{99}Tc^m$ activity was added to the water. The concentration of the microspheres was approximately 1.5 10^6 mL^{-1}, and the activity of ^{113}Sn was approximately 1.1 MBq. All values used in this study have been corrected for the half-life of the isotopes.

Sixteen mussels were given *S. typhimurium* labeled in the presence of 80 μg SnF_2 (designated $^{99}Tc^m$:80), and 16 mussels were given *S. typhimurium* labeled in the presence of 800 μg SnF_2 (designated $^{99}Tc^m$:800). Within each group of mussels, 50% were given ^{113}Sn-labeled microspheres (NEN TRAC microspheres, Du Pont) together with the bacteria. The nondegradable microspheres were made from styrene-divinyl benzene resins and were uniform in size (15 μm) (designated ^{113}Sn:ms).

Each mussel was exposed to radiolabeled microbes/microspheres for 5 hours. During this time the radioactivity was continuously recorded by the gamma camera. Then the mussel was carefully rinsed and repositioned in front of the camera, but now in clean seawater for recording of elimination of radioactivity. Finally, the mussel was dissected and the radioactivity of the organs and tissue fractions was measured using a well-shielded NaI(Tl) detector (15 cm in diameter; Nuclear Enterprises, UK) in a low-activity laboratory. The dissected fractions were the fimbriae part of the mantle, one pair of gills, one pair of palps, pericardial gland including the pericardium, gonad, one pair of kidneys, digestive

gland, terminal part of intestine, crystalline style, mantle, posterior adductor muscle, foot, anterior adductor muscle, and retractor muscle. The digestive gland was transected to distinguish the anterior part, including the esophagus, stomach, and diverticular folds (designated the stomach) from the posterior part, including the direct and recurrent intestine and the blind sac (designated the liver). The terminal intestine (designated the gut) was dissected separately. The dissected part of the kidneys was one of the longitudinal canals that lie on either side of the body at the root of the gills and the closest connected tissue (designated the renal). As the transit time of the radioactive particles in the mussel tissue was unknown, the dissection was done with different time lags, and these were randomized among the mussels. Meanwhile, the mussels were stored as under the pre-experimental conditions, with a daily exchange of water but without any food supply. The time lags were 5, 20, 28, and 54 hours for the mussels fed on $^{99}Tc^{m}$:80 ($n = 4$ in each group). The mussels fed on $^{99}Tc^{m}$:800 were dissected after 5 ($n = 4$), 20 ($n = 4$), 28 ($n = 2$), 54 ($n = 2$), and 68 ($n = 4$) hours. It should be pointed out that these differences in numbers of mussel dissected at different times were not planned but were a result of the time-consuming dissection.

Gamma Camera Technique

The gamma camera technique (MAXI II General Electric, Hermes System NuD, Nuclear Diagnostic, Hägersten, Sweden) was used. The camera continuously visualizes the distribution of radioactivity in the mussel. Furthermore, by outlining the regions of interest (ROIs) on the screen, the radioactivity in the region was quantified and listed and displayed as curves of activity versus time. The ROIs chosen in this study were the images of the stomach and gut regions. The parameters were calculated from the stomach region as follows:

Uptake = the fraction (%) of the initial amount of the given radioactivity that was accumulated when the maximum value in the ROI was reached. The initial radioactivity in the beaker represented the given activity. The maximum value was normalized to the initial radioactivity to avoid differences due to variations in the given activity, differences due to the distance between the mussel and the camera, and differences in the geometry of the mussels.

Elimination = the reduction (%) from the maximum value measured in the ROI. The reduction was determined after 20 hours of measurement. These values were normalized to the maximum value in the ROI to avoid differences in the uptake capacity.

Statistical Analysis

The influence on the uptake and the elimination of the bacteria due to the chemical treatments (80 or 800 μg SnF_2) and to the presence or absence of ^{113}Sn:ms was analyzed using two-way analysis of variance (ANOVA) and a Tukey test to allow multiple comparisons (Zar 1995). The variance of the uptake and elimination of $^{99}Tc^{m}$:80, $^{99}Tc^{m}$:800, and ^{113}Sn:ms was analyzed using one-way ANOVA and a Tukey test (Zar 1995). To obtain independent measurements for the ANOVA analysis, the $^{99}Tc^{m}$:80 and $^{99}Tc^{m}$:800 groups included mussels fed exclusively on bacteria, and the ^{113}Sn:ms group included the same numbers of individuals randomized from the mussels fed simultaneously on bacteria and microspheres.

Because of the differences between $^{99}Tc^{m}$:80 and $^{99}Tc^{m}$:800 in time lags before dissection, some of the mussels were excluded to equalize the groups when analyzing the distribution of the microbes in the mussel tissue. The excluded mussels were the four fed on $^{99}Tc^{m}$:800 dissected after 68 h and two mussels randomly chosen among those fed on $^{99}Tc^{m}$:80 dissected after 28 and 54 h, respectively. The Mann-Whitney rank sum test (Sokal & Rohlf 1995) was used to compare variances in content of radioactivity in the different fractions. Pearson product moment correlation (Snedecor & Cochran 1989) was used to investigate whether an increased amount of radioactivity in mussel tissue outside the digestive tract was related to a decrease in the digestive gland. In all of the statistical analyses, Sigma Stat version 2.0 (Jandel Scientific Software, San Rafael, CA) was used.

RESULTS

Viability of ^{99m}Tc-Labeled Bacteria

The viability of the unlabeled bacteria was 96.4 ± 1.4%. The viability of $^{99}Tc^{m}$:80 was 95.9 ± 1.7%, and for $^{99}Tc^{m}$:800 it was 89.6 ± 4.2%. The microscopic inspections showed the same size and shape of the bacteria compared with the unlabeled bacteria, and no aggregates were observed.

Uptake and Elimination of Radiolabeled Bacteria and Microspheres in M. edulis

When mussels were given *S. typhimurium* labeled with a small amount of stannous fluoride ($^{99}Tc^{m}$:80; Fig. 1A), as well as radioactive microspheres (^{113}Sn:ms; Fig. 1B), the uptake of bacteria in the stomach was slow and small and in the gut it was nearly inconspicuous, whereas the microspheres rapidly accumulated in the stomach and later in the gut. The uptake when $^{99}Tc^{m}$:80 was tested alone (Fig. 1C) was similar to that of the bacteria in the mixture (Fig. 1A). Mussels given bacteria labeled with more stannous fluoride ($^{99}Tc^{m}$:800; Fig. 2A) as well as ^{113}Sn:ms (Fig. 2B) showed similar uptake kinetics for the two kinds of particles. A similar pattern appeared for $^{99}Tc^{m}$:800 alone (Fig. 2C).

Two-way ANOVA confirmed that the presence of the ^{113}Sn:ms affected neither the uptake nor the elimination of $^{99}Tc^{m}$:80 and $^{99}Tc^{m}$:800, but the difference due to the amount of stannous fluoride used for the labeling of the bacteria was significant (Table 1). The uptake (Fig. 3) and elimination (Fig. 4) varied on an individual basis. Still, the statistical analysis showed that the variances in the processing of $^{99}Tc^{m}$:80 compared with $^{99}Tc^{m}$:800 and ^{113}Sn:ms were significant. The uptake of ^{113}Sn:ms and of $^{99}Tc^{m}$:800 was significantly higher than that of $^{99}Tc^{m}$:80 [one-way ANOVA, $F = 32.4$; degrees of freedom (df) between subjects = 7; $P < 0.001$, post hoc Tukey test]. Similarly, the elimination of ^{113}Sn:ms and of $^{99}Tc^{m}$:800 was significantly higher compared with that of $^{99}Tc^{m}$:80 (one-way ANOVA, $F = 20.8$, df between subjects = 5; $P < 0.001$, post hoc Tukey test).

The Distribution of Radioactivity in M. edulis

The radioactivity in the different organs and tissues showed great differences between individuals. Despite this, the differences between the three different groups ($^{99}Tc^{m}$:80, $^{99}Tc^{m}$:800, and ^{113}Sn:ms) were pronounced. Most of the activity of ^{113}Sn:ms in the digestive tract (Fig. 5) was recovered in the stomach, in the liver, and in the gut. The activities of $^{99}Tc^{m}$:80 and $^{99}Tc^{m}$:800 were even higher in the stomach and liver. $^{99}Tc^{m}$:80 could hardly be detected in the gut. The gills contained significantly more

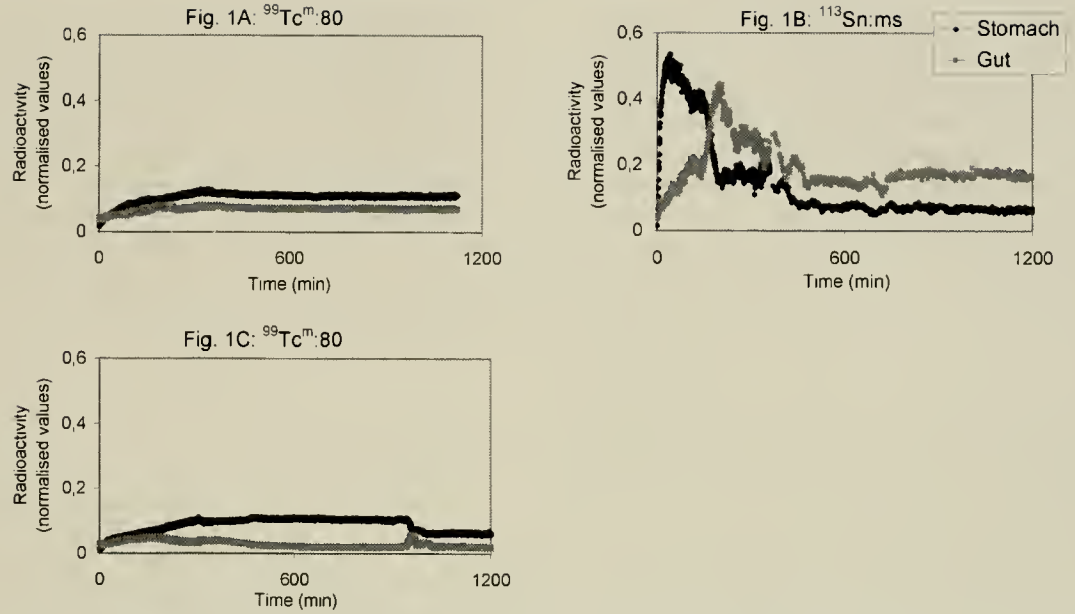

Figure 1. Chart lines showing the radioactivity measured in the ROIs (stomach and gut) in a mussel which was simultaneously given *S. typhimurium* labeled with $^{99}Tc^{m}$ in the presence of 80 µg SnF_2 ((A) $^{99}Tc^{m}$:80) and microspheres labeled with ^{113}Sn ((B) ^{113}Sn:ms). (C) Shows a mussel which was given $^{99}Tc^{m}$:80 only. The amount of radioactivity is normalized to the initial amount given to the mussel.

$^{99}Tc^{m}$:80 than $^{99}Tc^{m}$:800 (ANOVA, df = 15, $P = 0.028$). There were low values of $^{99}Tc^{m}$, and ^{113}Sn:ms were almost undetectable in the mussel tissue outside the digestive tract. When analyzing fractions with detectable activity, the gonad, posterior adductor muscle, mantle, and renal showed significantly (Mann-Whitney rank sum test) higher activities of $^{99}Tc^{m}$:80 than of $^{99}Tc^{m}$:800 (Table 2). The correlation analysis (Table 3) showed that when the amount of $^{99}Tc^{m}$:80 in the digestive gland decreased, the amount in gonad, adductor muscle, mantle, and renal increased. This was not the case when comparing the corresponding values for $^{99}Tc^{m}$:800.

The Transit Time of $^{99}Tc^{m}$ in M. edulis

The number of mussels dissected at 5, 20, 28, and 54 h were too few for any statistical ANOVA of transit time, but the general

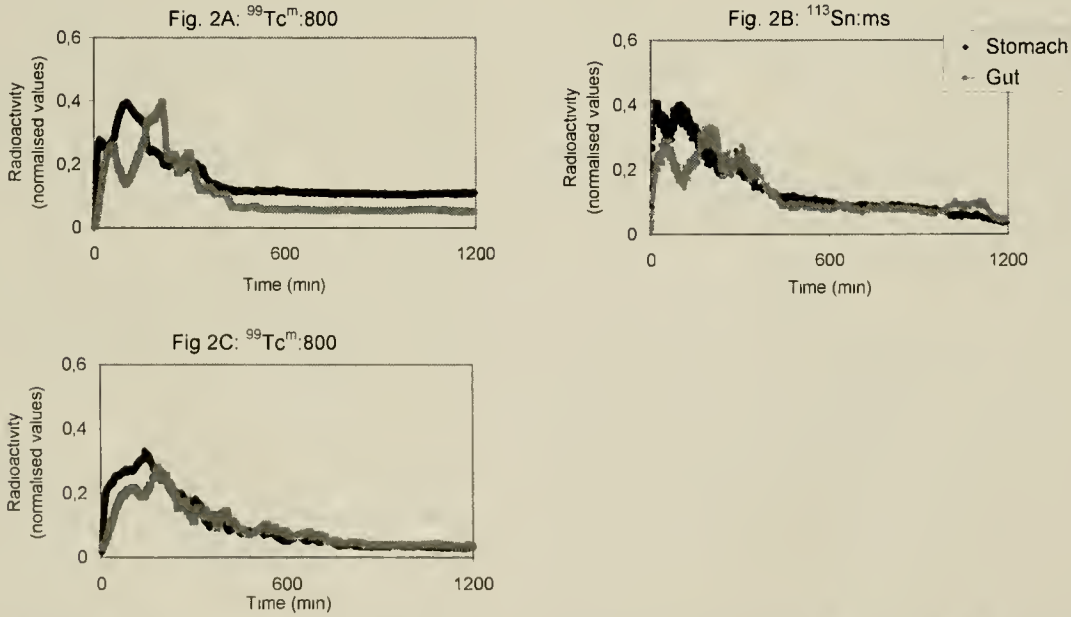

Figure 2. Chart lines showing the radioactivity measured in the ROIs (stomach and gut) in a mussel which was simultaneously given *S. typhimurium* labeled with $^{99}Tc^{m}$ in the presence of 800 µg SnF_2 ((A) $^{99}Tc^{m}$:800) and microspheres labeled with ^{113}Sn ((B) ^{113}Sn:ms). (C) Shows a mussel which was given $^{99}Tc^{m}$:800 only. The amount of radioactivity is normalized to the initial amount given to the mussel.

TABLE 1.

Two-way ANOVA table, comparing the influence on the uptake and the elimination of *S. typhimurium* in *M. edulis* due to the chemical treatments of the bacteria ($^{99}Tc^{m}$:80 or $^{99}Tc^{m}$:800) and in the absence or presence of the microspheres in the medium (mono- or multiple medium).

	df	SS	F
Source of variation for uptake			
Chemical treatment ($^{99}Tc^{m}$:80 or $^{99}Tc^{m}$:800)	1	6080	39.219***
Mono- or multiple medium	1	0.0166	0.0001 (NS)
Residual	28	4341	
Total	31	1053	
Source of variation for elimination			
Chemical treatment ($^{99}Tc^{m}$:80 or $^{99}Tc^{m}$:800)	1	6698	16.848***
Mono- or multiple medium	1	1021	2.568 (NS)
Residual	(20) 21	8348	
Total	(23) 24	16,384	

*** $P < 0.001$.
NS, not significant; $P > 0.5$.
df, degrees of freedom; SS, sum of squares.

patterns based on the mean values in Figure 6 gave some indications. In the posterior adductor muscle and the mantle, there was a reduction of radioactivity from $^{99}Tc^{m}$:80 and $^{99}Tc^{m}$:800 after 28 h. The most rapid accumulation and the highest values were measured in the gonads of the mussels fed $^{99}Tc^{m}$:80. The amount of $^{99}Tc^{m}$:80 did not decrease in the gonad and the renal during 54 h. The mussels fed on $^{99}Tc^{m}$:800 did not show any reduction in the renal after 68 h (Fig. 6). In the digestive tract, there was also detectable activity after 54 h. In the digestive gland, there was 0.45 ± 0.26 MBq × g^{-1} for $^{99}Tc^{m}$:80 and 0.57 ± 0.03 MBq × g^{-1} for $^{99}Tc^{m}$:800. In the gills, there was 0.16 ± 0.06 MBq × g^{-1} from $^{99}Tc^{m}$:80 but not detectable values from $^{99}Tc^{m}$:800.

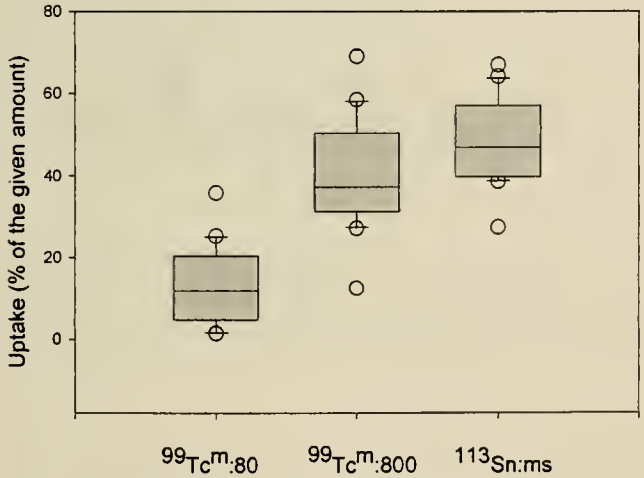

Figure 3. The uptake of radioactivity in the stomach (% of the given amount) when its maximum activity was measured, in 16 mussels fed on *S. typhimurium* labeled with $^{99}Tc^{m}$ in the presence of 80 and 800 µg SnF_2, respectively (^{99m}Tc:80 and ^{99m}Tc:800) and the microspheres labeled with ^{113}Sn (^{113}Sn:ms). Box plots display the median of the data, the lower and upper quartiles, and the lowest and highest values observed.

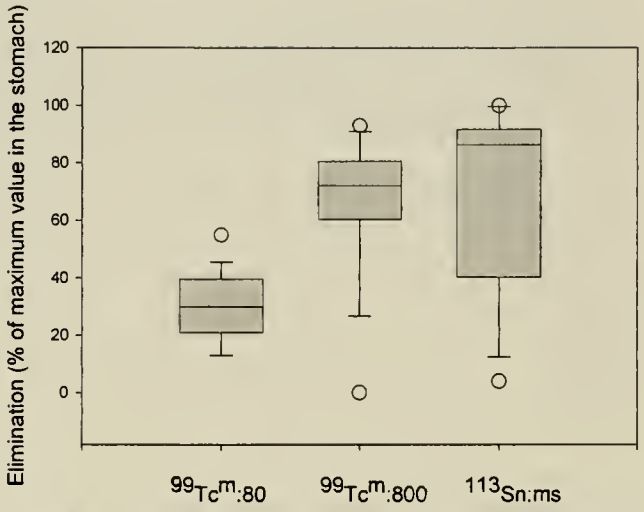

Figure 4. The elimination of radioactivity (% of the maximum value in stomach) determined 20 h after the experiment started, in 12 mussels fed on *S. typhimurium* labeled with $^{99}Tc^{m}$ in the presence of 80 and 800 µg SnF_2, respectively (^{99m}Tc:80 and ^{99m}Tc:800) and the microspheres labeled with ^{113}Sn (^{113}Sn:ms). Box plots display the median of the data, the lower and upper quartiles, and the lowest and highest values observed.

DISCUSSION

S. typhimurium 395 M R10 is an Rd-mutant deficient of the 0-antigenic polysaccharide side chain and with a pronounced negative surface charge (Edebo et al. 1980). Hernroth et al. (2000) described the chemical manipulation of *S. typhimurium*, using stannous fluoride. The electrophoretic mobility toward a cathode was significantly reduced for $^{99}Tc^{m}$:800 when compared with $^{99}Tc^{m}$:80 and untreated bacteria. In this study we have found differences in the mussel processing of the differently manipulated bacteria.

The preingestive selection of particles is expected to take place on the gills or on the labial palps. The structure of the gills is known to divert particles due to size (Riisgård et al. 1996), and it

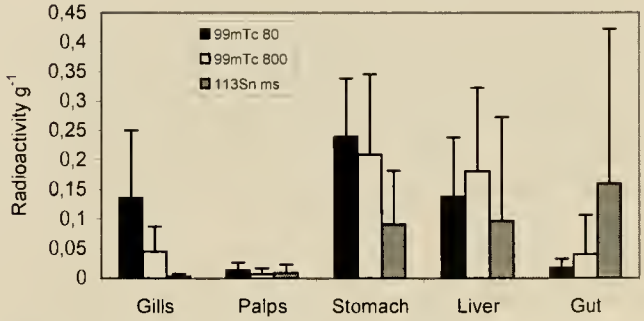

Figure 5. Distribution of radioactivity of *S. typhimurium* (+ standard deviation) labeled with $^{99}Tc^{m}$ in the presence of 80 and 800 µg SnF_2, respectively (^{99m}Tc:80 and ^{99m}Tc:800) and microspheres labeled with ^{113}Sn (^{113}Sn:ms), in the digestive tract [gills, labial palps, posterior part of the digestive gland (stomach) and the anterior part (liver) and terminal intestine (gut)]. The columns are based on the mean values (per g tissue) from 12 mussels dissected within 54 h.

TABLE 2.

Mann-Whitney rank sum test: comparison of median values ($^{99}Tc^m \times mg^{-1}$) in posterior adductor muscle, mantle, gonad, and renal from mussels fed on *S. typhimurium*, labeled in the presence of 80 and 800 µg of SnF_2, respectively.

Group	*n*	Median	25%	75%	$P < 0.05$
Adductor:80	12	0.99	0.47	2.35	
Adductor:800	12	0.29	0.15	0.55	Yes
Mantle:80	12	4.67	1.53	6.23	
Mantle:800	12	1.58	0.37	2.61	Yes
Gonad:80	12	1.5	0.85	3.22	
Gonad:800	12	0.63	0.12	1.00	Yes
Renal:80	12	42.5	27.5	59.5	
Renal:800	12	17.4	5.36	30.1	Yes

has been suggested that potentially nutritive particles will be selected relative to inert particles on the labial palps (Hylleberg & Gallucci 1975, Newell & Jordan 1983, Bayne et al. 1993). The uptake of $^{99}Tc^m$:800 and ^{113}Sn:ms was similar and much faster than that of $^{99}Tc^m$:80, showing that the size alone did not determine the uptake, because the $^{99}Tc^m$-labeled bacteria were much smaller (approximately 1µm) than the microspheres (15 µm). The higher uptake capacity of $^{99}Tc^m$:800 was correlated to a decrease of the net negative cell surface charge, indicating that negative charge might antagonize uptake. This study showed discrimination in ingestion of the less manipulated bacteria, and significantly more bacteria of this kind were "trapped" on the gills. A proper explanation to this requires further studies, and we suggest that it should include electrostatic repulsion and also hemocytic attachment or engulfment of bacteria on the gills.

The elimination of the bacteria was also affected by the chemical modification of the cell surface. The elimination was significantly lower for $^{99}Tc^m$:80 than that for $^{99}Tc^m$:800 which again was similar to that of ^{113}Sn:ms. The less-modified bacteria were to a high degree accumulated in the stomach part of the digestive gland, but were hardly present in the gut. According to Birkbeck and MacHenery (1982), this indicates a postingestive selection based on phagocytic activity. These authors showed, in their study of *M. edulis*, that the processing of bacteria after phagocytic uptake in the hepatopancreas digested the bacteria into polymers that were

TABLE 3.

Pearson Product Moment Correlation table from dissected fractions of the mussel tissue. The relationship between the contents of radioactivity (over time, as described in Materials and Methods) in the digestive gland and the gonad, posterior adductor muscle, mantle, and renal in mussels ($n = 12$) fed on *S. typhimurium* labeled in the presence of 80 and 800 µg SnF_2 (marked as :80 and :800), respectively.

	Gonad:80	Adductor:80	Mantle:80	Renal:80
Digestive gland:80	−0.696**	−0.898***	−0.846***	−0.825***
	Gonad:800	**Adductor:800**	**Mantle:800**	**Renal:800**
Digestive gland:800	−0.356 (NS)	−0.395 (NS)	−0.095 (NS)	−0.244 (NS)

** $P < 0.01$.
*** $P < 0.001$.
NS, not significant; $P > 0.05$.

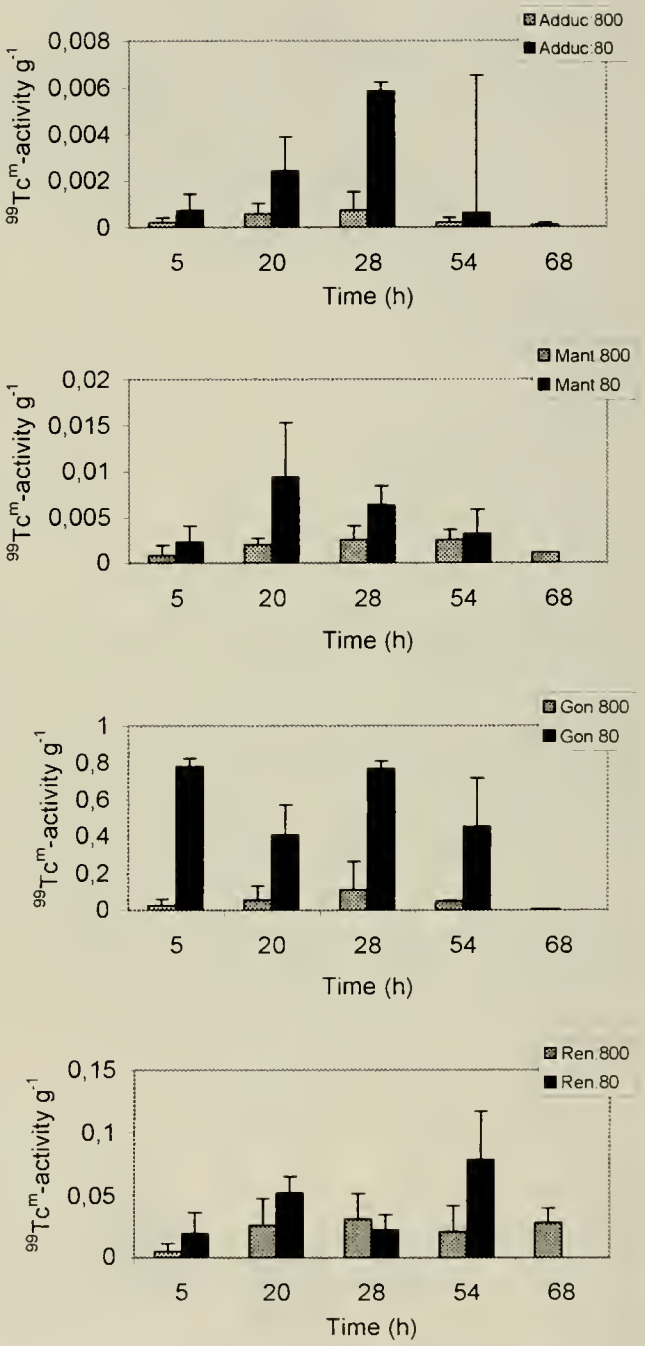

Figure. 6. Distribution of radioactivity of *S. typhimurium* labeled with $^{99}Tc^m$ in the presence of 80 and 800 µg SnF_2, respectively ($^{99}Tc^m$:80 and $^{99}Tc^m$:800) in posterior adductor muscle (Adduc), mantle (Mant), gonad (Gon), and renal (Ren) (NB: different scales). The columns are based on the mean values (per g tissue) from mussels dissected after 5, 20, 28, 54, and 68 h.

transferred to other sites in the mussel, whereas most of the lysozyme-resistant bacteria were rejected as fecal production. It has previously been shown that *M. edulis* can lyse bacteria extracellularly (Prieur 1981), but the slow processing and the preferential absorption of $^{99}Tc^m$:80 compared with $^{99}Tc^m$:800 that evidently occurred in our study indicated a predominance of phagocytosis and intracellular digestion of the less-manipulated bacteria. Absorption of radioactivity from $^{99}Tc^m$:80 was supported by the relationship between the decrease of radioactivity in the digestive

gland and the appearance in organs and tissue outside the digestive tract. This was not found for $^{99}Tc^{m}$:800. These bacteria were less absorbed and were more directly transferred into the intestine. The faster elimination with lower absorption efficiency shown for $^{99}Tc^{m}$:800 and ^{113}Sn:ms indicated extracellular digestion.

Radioactivity was still detectable in the digestive tract of the mussels dissected after 54 hours. This observation was made in a closed system in which the water was exchanged only on a daily basis and should not be compared with the depuration study of Martinez-Manzanares et al. (1992). They showed a rather rapid elimination of *Salmonella* spp. after purification in running seawater. However, Plusquellec et al. (1994) managed to detect *Salmonella* spp. in air-stored mussels, 20 days after contamination. Minet et al. (1995) found culturable cells of *S. typhimurium* in the gut 1 week after contamination. The possibility for extracellular survival of *S. typhimurium* in the digestive tract, including the gills, as indicated by the presence of radioactivity after 54 hours, needs further investigation. Extracellular survival can thus be a cause for shellfish-borne gastrointestinal infections and should be included in risk assessment. Likewise, we found ^{99m}Tc distributed in mussel tissue outside the digestive tract, such as gonads, kidney, mantle, and adductor muscle, 1-2 days after exposure to the bacteria. This might be caused by degraded bacteria but might also be an effect of resistance to phagocytosis and killing.

In the previous study by Hernroth et al. (2000), the stability of the isotope bindings to *S. typhimurium* in seawater was not significantly different for $^{99}Tc^{m}$:80 and $^{99}Tc^{m}$:800. Fragile binding could increase the amount of hydrolysed, reduced technetium or free pertechnetate, but these radiochemical impurities did not influence the uptake capacity of $^{99}Tc^{m}$ in the mussel. The possibility of diffusion of released $^{99}Tc^{m}$ into the mussel tissue could, as pointed out, interact with the measurement of the distribution of radioactivity in the mussel tissue. However, as the binding stability of $^{99}Tc^{m}$ was comparable for $^{99}Tc^{m}$:80 and $^{99}Tc^{m}$:800, this could not explain the differences in the distribution of these microbes shown in this study.

The viability estimated with the fluorescent probe of the labeled *S. typhimurium* was initially good (96% for $^{99}Tc^{m}$:80 and 90% for $^{99}Tc^{m}$:800), and the microscopic inspections confirmed intact cell size and shape and no aggregates. Thus, it was considered that the same numbers of viable $^{99}Tc^{m}$:80 and $^{99}Tc^{m}$:800 were given to the mussels. The differences in the uptake between $^{99}Tc^{m}$:80 and $^{99}Tc^{m}$:800 occurred directly from start, indicating that viability was not the discriminating factor for uptake.

This investigation has shown that the uptake, distribution, and elimination of microbes by the blue mussel are strongly influenced by the cell surface characteristics of the microbe. This factor might be at least as important as particle size. We suggest that recognition for phagocytic uptake might play an important role in the processing of microbes.

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ON THE POSSIBILITY OF USING RADIOACTIVE LABELING AND GAMMA CAMERA TECHNIQUE TO STUDY *SALMONELLA TYPHIMURIUM* IN THE BLUE MUSSEL, *MYTILUS EDULIS*

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ABSTRACT This paper presents a radiolabeling method for *Salmonella typhimurium* to be used for *in vivo* studies on the kinetics of uptake in blue mussels with a gamma camera technique. *S. typhimurium* bacteria were labeled with technetium-99m ($^{99}Tc^{m}$) at conditions preserving the viability in seawater and the cell surface properties of the bacteria. Stannous fluoride (SnF_2) was used to facilitate the binding of $^{99}Tc^{m}$ to the bacteria. The toxicity of SnF_2 could inhibit the growth of bacteria, and it can also bind extracellularly and reduce the negative cell surface charge of the bacteria. Additionally, SnF_2 can cause radiochemical by-products such as Tc-stannous colloids, which might interact with the image analysis. To optimize the labeling, two different concentrations of SnF_2 used in the labeling process were evaluated. Neither the efficiency nor the stability of the binding of $^{99}Tc^{m}$ to the bacteria increased significantly, when the amount of SnF_2 was increased 10-fold. Both treatments of bacteria reduced the viable counts, whereas the viability assessed microscopically with fluorescent probe was affected only little. However, after incubation in seawater, the viability was reduced for cells treated with the highest concentration of SnF_2. Still, approximately 60% remained viable. Presence of radioactivity, not bound to bacteria, was investigated by centrifugation in Percoll. Less than 4% of probable Tc-stannous colloids were found, and they were therefore not considered to be disturbing the imaging analysis. The net negative surface charge of the bacteria, examined by measuring the electrophoretic mobility, was significantly reduced when the concentration of SnF_2 increased, but was still negative. Radioactive particles, formed by mixing $^{99}Tc^{m}$ and SnF_2 in the absence of bacteria, were rapidly taken up by mussels in a way similar to that of the more heavily labeled bacteria. When less SnF_2 was used for labeling of the bacteria, different uptake and processing kinetics were seen. Thus, to keep the natural conditions, the concentration of the labeling compounds have to be minimized. The study showed that it is possible to balance the labeling method and get a valuable tool for following the uptake and fate of *S. typhimurium* in blue mussels.

KEY WORDS: Gamma camera, radiolabeling, 99m-technetium, ^{99m}Tc, *Salmonella typhimurium*, bacteria, *Mytilus edulis*, bivalves, molluscs, uptake, elimination, enteric infections

INTRODUCTION

The blue mussel, *Mytilus edulis*, is in nature exposed to a mixture of particles and is able to accumulate high numbers of microbes from the surrounding water. The potential for the mussel to become a carrier of food-borne diseases is therefore significant, and several reports have pointed out the molluscs as commonly incriminated in outbreaks of enteric infections (West et al. 1985, Martinez-Manzanares et al. 1992, Wittman and Flick 1995, Ripabelli et al. 1999). Depuration studies on bivalves have shown great individual variations between mussels (Heffernan and Cabelli 1971, Plusquellec et al. 1994) and variations due to bacteria species (Plusquellec et al. 1998). This stresses the necessity of studies on the uptake and elimination of microorganisms in individual bivalves to establish satisfying monitoring programs and to improve risk assessment with respect to public health.

Gamma-emitting radionuclides in bacteria can be used for *in vivo* imaging distribution. Technetium-99m ($^{99}Tc^{m}$) isotope has been used as a radiotracer when studying phagocytosis of viable bacteria in vertebrates (Plotkowski et al. 1987, Bernardo-Filha et al. 1991, Perin et al. 1997). In the coupling process, stannous fluoride (SnF_2) has been used to reduce $^{99}Tc^{m}$ from +VII to +IV, which facilitates its binding (Rhodes 1991, Perin et al. 1997). SnF_2 is also known for its bactericidal effect (Tsao et al. 1982, Caufield et al. 1987, Oosterwaal et al. 1989, Oosterwaal et al. 1991), and therefore the amount used in the labeling process has to be selected with care. SnF_2 acts as an inhibitor of glycolytic enzymes, as it binds to SH groups. The metal ions interact with both Gram-positive and Gram-negative bacteria, and the antimicrobial effect depends on the concentration of free ions as well as the chemistry of the ions in the specific system (Scheie 1994). In addition, the concentration of SnF_2 must be used with care, as it might influence the cell surface properties of the bacteria. Olsson and Oldham (1978) have proved that the binding of metal ions to bacteria alters their cell surface charge and adherence ability. With the amount of stannous fluoride used by Plotkowski et al. (1987) in the $^{99}Tc^{m}$ labeling of the *Pseudomonas aeruginosa*, the electrophoretic mobility was not changed, but Perin et al. (1997) showed that the $^{99}Tc^{m}$ labeling of *S. abortusovis* demands a higher concentration of SnF_2. As it has been shown that particle processing in *M. edulis* can be effected when the particles are enriched in metals (Wang et al. 1995, Al-

lison et al. 1998), the concentration of stannous in the labeling process is critical.

An indirect labeling method has been used to follow the distribution of leukocytes in humans (Mock and English 1987, Puncher and Blower 1995). In these assays, leukocytes were labeled intracellularly by phagocytosis of colloidal compounds of $^{99}Tc^{m}$ and SnF_2 (Tc-stannous colloid). These studies also demonstrated that active compounds might have occurred as nonspecific, cell surface-bound labeling with low affinity and soluble radiocolloids. Radiochemical by-products might also occur in the direct labeling of bacteria. As a side reaction, the reduced technetium can bind to more low-affinity binding sites or together with SnF_2 form Tc-stannous colloids. There is a possibility that released pertechnetate or Tc-stannous colloids might be ingested by the mussel and confuse the image analyses of the bacteria. Because of the complications described, the labeling method has to be optimized to avoid decreased viability of the bacteria and radiochemical impurities. Furthermore, alteration of the surface properties by the labeling process has to be taken into consideration, as this could affect the processing of the bacteria in *M. edulis*.

Salmonella can appear in the marine environment because of fecal contamination (Prazeres Rodrigues et al. 1989, Papapetropoulou & Moschopoulos 1996, Wilson & Moore 1996) and is of great interest in terms of shellfish safety. The aim of this study was to investigate and evaluate the $^{99}Tc^{m}$-labeled *Salmonella typhimurium* as a tool to study its uptake and fate in *M. edulis*. *S. typhimurium* 395 MR10 was chosen because it is nonvirulent and known to be well accessible to phygocytosis, killing, and degradation in mammalian systems (Edebo et al. 1980). Considering the evaluation of the method used in the direct labeling of *S. abortusovis* (Perin et al. 1997), we compared the effect of two different concentrations of SnF_2 on (1) the viability of labeled *S. typhimurium* in seawater, (2) the labeling efficiency of the bacteria and the stability of the label in seawater, (3) the formation of Tc-stannous colloids during the labeling process, (4) the cell surface charge of the labeled bacteria, and (5) interaction of the labeled bacteria with *M. edulis*.

MATERIALS AND METHODS

Bacterial strain and growth conditions

S. typhimurium 395 MR10 (chemotype Rd, deficient of *O*-antigenic polysaccharide side chain and most core sugars of the lipopolysaccharide) has been described by Edebo et al. (1980). A single bacterial colony was harvested from a nutrient agar plate (beef extract, Oxoid 10 g; peptone, Oxoid 10 g; NaCl 8 g; glucose 7.5 g; and agar 1.4%) and cultured in glucose broth, pH 7.0-7.2 (Lindberg et al. 1970) at 37 °C on a rotary shaker (200 rpm) for 16 h. The bacteria were washed three times by centrifugation (2000 rpm, 10 min, 4 °C) in 0.9% NaCl and resuspended in 2 mL 0.9% NaCl (2.5×10^9 mL^{-1}). With these culturing conditions, the bacteria are considered to reach the prestationary phase.

Radiolabeling of bacteria

One milliliter of the bacterial suspension (2.5×10^9 mL^{-1}) was incubated with approximately 50 MBq $^{99}Tc^{m}$-pertechnetate and 2 mL of 37 °C 0.9% NaCl containing 80 and 800 μg SnF_2, respectively, to cause reduction of $^{99}Tc^{m}$. After incubation for 20 min at 37 °C on a rotary shaker (200 rpm), the bacteria were centrifuged and washed three times. Ascorbic acid (0.25 mg × mL^{-1}) was added to the NaCl to prevent reoxidation of the isotope (Rhodes 1991). The bacteria were resuspended in 1 mL 0.9% NaCl.

Bacterial viability

For estimation of the effect of the labeling procedure on the viability, bacteria treated with $^{99}Tc^{m}$-pertechnetate as well as with 80 (n = 6) or 800 (n = 6) μg SnF_2, respectively, were compared with control bacteria incubated in 2 mL 0.9% NaCl (n = 6) only. The suspensions of bacteria were serially diluted in phosphate-buffered saline (NaCl 0.15 M, sodium phosphate 0.01 M, pH 7.2 7.4), spread onto nutrient agar plates using a spiral plating system (Spiral System Inc., Cincinnati, OH), incubated at 37 °C for 24-48 h, and the colonies counted and the colony-forming units per mL (CFUs × mL^{-1}) calculated.

The viability of the bacteria was also investigated using the LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit (Molecular Probes, The Netherlands). Live bacteria appear with green fluorescence (SYTO 9), whereas the red fluorescence of membrane-impermeant propidium iodide dominates membrane-compromised bacteria (Haugland 1996). The bacterial suspensions were diluted ($5 \times 10^6 \times mL^{-1}$) in sterile filtered (Schleicher & Schuell, Keene, NH; FP 030/3) seawater (33.69 PSU, 6 °C), incubated on a rotary shaker (200 rpm) with the fluorescent probe for 15 and 180 min, observed in an epifluorescence microscope (Zeiss Axioscop, excitation filter BP450-490, dichroic reflector 510, barrier filter LP5159, Zeiss, Germany), and the fraction of viable cells calculated. After all labeling processes, the bacterial suspensions were observed in a light microscope (12.5 × 100 times magnification) to check possible effects on shape and size and aggregate formation.

Efficiency and stability of the $^{99}Tc^{m}$ labeling

After the labeling process, the radioactivity of $^{99}Tc^{m}$-labeled bacteria was measured using a well-shielded NaI(Tl) detector (15 cm[diameter]; Nuclear Enterprises, UK) in a low-activity laboratory. The labeling efficiency was expressed as percentage of the initial activity bound.

The stability of the binding was tested by incubation of three batches of bacteria (final concentration $5 \times 10^6 \times mL^{-1}$), labeled in the presence of 80 and 800 μg SnF_2 respectively, in filtered (Millipore 0.3 μm) seawater (33.69 PSU, 6 °C). Triplicate samples were taken within 3 min and then after 15, 30, 60, and 180 min. Particles >0.2 μm were separated from the water using sterile filters (Schleicher & Schuell; FP 030/3), and the filtered volume was collected in vials and the fractions were measured in the well-shielded NaI(Tl) detector. The bounded part was expressed as the particulate fraction of the total radioactivity.

Radiochemical by-products

Possible formation of Tc-stannous colloids in the labeling solution was investigated by separation in Percoll (n = 3), with a density of $1.12 \text{ g} \times mL^{-1}$. One milliliter of the labeled bacteria was layered on the Percoll, and the tubes were centrifuged for 20 min at 2000 rpm. Free $^{99}Tc^{m}$-pertechnetate and two colloidal suspensions were used as references. The colloidal suspensions were formed when $^{99}Tc^{m}$ was incubated in the presence of 80 and 800 μg SnF_2, respectively, without bacteria (in this paper called Tc+80 and Tc+800). After centrifugation, the tubes were placed in front of the gamma camera and the separated parts were measured and calculated as a percentage of the total radioactivity.

Cell microelectrophoresis

The cell surface charge of the bacteria, labeled in the presence of 80 and 800 μg SnF_2, respectively, was investigated using microelectrophoresis (Mark II, Rank Brothers Ltd., Cambridge, England). The electrophoretic mobility ($m^{-2} \times V^{-1}s^{-1}$) of the chemically treated bacteria was compared with that of untreated bacteria. The bacteria were diluted in 5 mM KCl, and the time needed to pass a 180-μm grid in the electric field (90 V; distance between electrodes, 64.6 cm) was measured 10 times. The variances between the treatments were analyzed using one-way analysis of variance on ranks (Student-Newman-Keuls method) (Sokal & Rolph 1969).

Uptake by M. edulis of radiolabeled bacteria and possible by-products

Mussels were kept in circulating seawater of approximately 33 PSU at 6 °C and fed the nanoflagellate *Isochrysis galbana* before the experiment started. The mean length of the mussels was 7.1 ± 0.5 cm, and the mean wet flesh weight was 9.1 ± 3.0 g. Two sets of two mussels each were used to study the uptake of *S. typhimurium* labeled in the presence of 80 μg SnF_2 (S:80) and in the presence of 800 μg SnF_2 (S:800). As control mussels, two were given the colloidal suspension (without bacteria) incubated with $^{99}Tc^m$ and 80 μg SnF_2 (Tc+80), two mussels were given the suspension incubated with $^{99}Tc^m$ and 800 μg SnF_2 (Tc+800), and two mussels were given a suspension with free $^{99}Tc^m$-pertechnetate (free Tc).

Single mussels were positioned in front of the gamma camera, and hung above the bottom in beakers containing approximately 700 mL of filtered (Millipore 0.3 μm) seawater (33.7 PSU). The water was kept at 6 °C, well mixed with a stirrer, and oxygenated during the experiment. Labeled bacteria or reference solutions were added to the beaker. The final concentration when given the bacteria was approximately 5×10^6 mL^{-1}. The distribution of radioactivity was visualized for 5 h using a conventional, computer-aided gamma camera technique (MAXI II, General Electric; Hermes-system NuD, Nuclear Diagnostic, Hägersten Sweden) as shown in Figure 1. By outlining the region of interest (ROI) of the image, the amount of radioactivity in the chosen region was measured. The ROI chosen for this study was the area where the radioactivity was accumulated after passing the gills, identified as the stomach. The uptake was estimated as the accumulated fraction of the given amount of radioactivity (%), measured when the maximum value in the ROI was reached and the uptake rate was calculated (% min^{-1}). The values were normalized to the initial amount of radioactivity to eliminate differences in the given amount of activity and geometric properties, such as mussel size and shape and the distance between the mussel and the camera. The radioactivity was corrected for the half-life of the isotope (6 h).

RESULTS

Viability of the ^{99m}Tc-labeled bacteria

Compared with the unlabeled bacteria, the viable counts on agar plates were significantly reduced for both S:80 and S:800. When analyzing the unlabeled bacteria, 187 ± 29 × 10^7 CFU were found. The CFUs for S:80 and S:800 were 33 ± 16 × 10^7 and 10 ± 2 × 10^7, respectively, corresponding to a reduction of 82 and 95%, compared with the control. The fluorescence assay showed (Fig. 2) that after incubation for 15 min in seawater, the viable

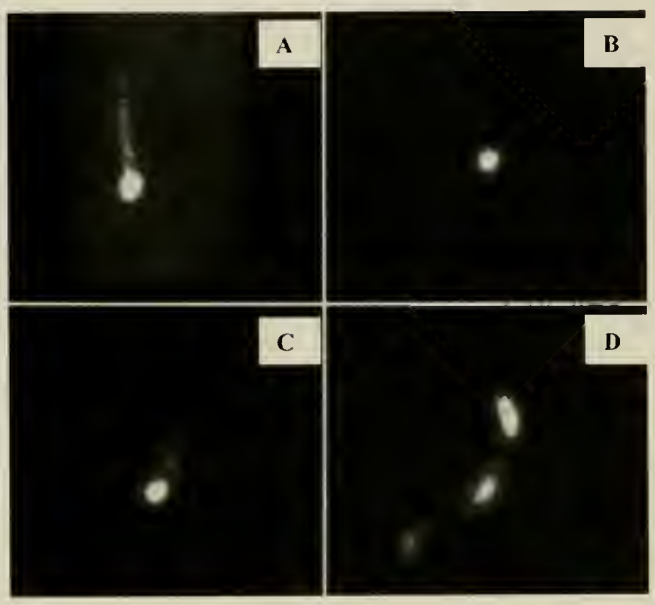

Figure 1. Gamma camera image of a mussel after being given bacteria labeled with $^{99}Tc^m$ in the presence of 800 μg SnF_2. (A) Concentrated along the gills of the mussel. (B) Accumulated in the stomach region. (C) Directed to the gut. (D) Transported as fecal content through the gut.

fraction of S:80 (88 ± 6%) was similar to that of the unlabeled bacteria (94 ± 2%). The corresponding value for S:800 was 81 ± 8%. After incubation for 180 min in seawater, the viable fractions of the unlabeled bacteria and S:80 remained unchanged, being 97.3 ± 0.6% for the unlabeled bacteria and 96.9 ± 2.2% for S:80, whereas for S:800 it was significantly reduced (59.2 ± 4.5%). The microscopic inspections showed that the cell size (approximately 1 μm) and shape were not altered for S:80 and S:800, and no aggregates were observed.

Efficiency and stability of the $^{99}Tc^m$ labeling

The efficiency of the labeling of S:80 (77 ± 7%) and of S:800 (70 ± 14%) was not significantly different. The amount of $^{99}Tc^m$ released during the first hour in seawater was approximately 37% for S:80 and 30% for S:800. During the following hour, the bound $^{99}Tc^m$ stayed more stable (Fig. 3).

Radiochemical by-products

After centrifugation in Percoll (Fig. 4), free Tc stayed on the top layer (96 ± 1%), as did the bacteria, S:80 (95 ± 2%), and S:800

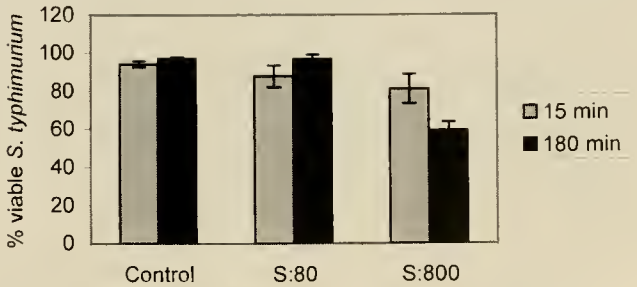

Figure 2. Viable cells (% of the total number of cells) (+SD, n=6), estimated by fluorescence assay, of *S. typhimurium* labeled in the presence of 80 (S:80) and 800 (S:800) μg SnF_2, respectively, and unlabeled *S. typhimurium* (Control). The viability was estimated after 15 and 180 min of incubation in seawater.

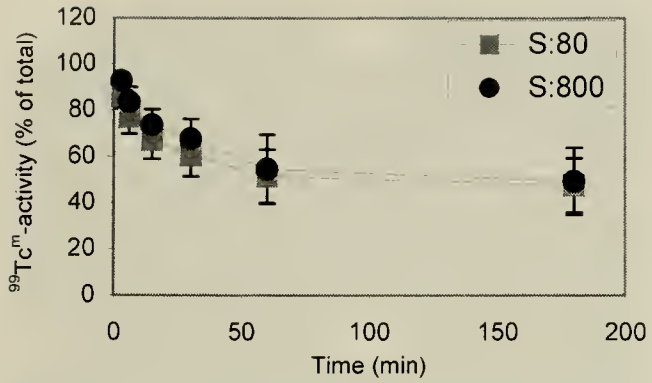

Figure 3. The particulate fraction (%) of the total amount of $^{99}Tc^{m}$ (+SD, n=9) from *S. typhimurium* labeled in the presence of 80 (S:80) and 800 (S:800) µg SnF_2, respectively, measured during 180 min of incubation in seawater.

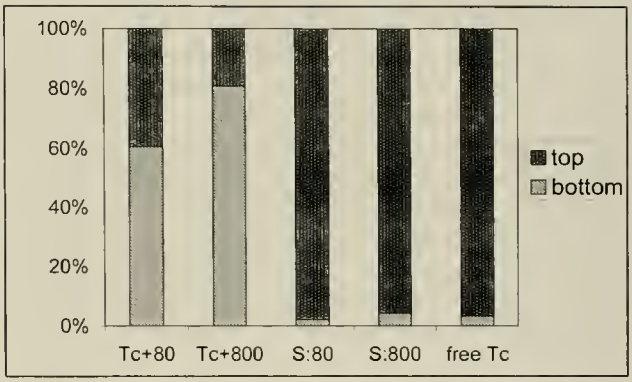

Figure 4. The mean percentage of the total amount of $^{99}Tc^{m}$ activity (n=3) accumulated in the top and the bottom layers of the test tube after centrifugation in Percoll. The columns show $^{99}Tc^{m}$ incubated only with 80 (Tc+80) and 800 (Tc+800) µg SnF_2, *S. typhimurium* labeled with $^{99}Tc^{m}$ in the presence of 80 (S:80) and 800 (S:800) µg SnF_2, and free $^{99}Tc^{m}$-pertechnetate (free Tc).

(92 ± 1%), and less than 4% were found at the bottom. In the tubes with colloids formed during the incubation of $^{99}Tc^{m}$ with SnF_2 (without bacteria), more were found at the bottom. The bottom fraction increased with the amount of SnF_2, showing 48 ± 17% of the radioactivity in Tc+80 and 71 ± 22% in Tc+800.

Cell microelectrophoresis

One-way analysis of variance on ranks (Kruskal-Wallis) showed that the electrophoretic mobility for *S. typhimurium* was affected by the treatments of the bacteria (Table 1). There was a statistically significant reduction in electrophoretic mobility for S:800 compared with S:80 and untreated bacteria. The median value for S:800 was 2.3×10^{-9} $m^2 \times V^{-1}s^{-1}$. For S:80 it was 5.4×10^{-9} $m^2 \times V^{-1}s^{-1}$ and for untreated bacteria 4.7×10^{-9} $m^2 \times V^{-1}s^{-1}$.

Uptake by M. edulis of radiolabeled bacteria and possible by-products

Preliminary studies on the uptake of labeled bacteria by mussels showed that initially the radioactivity accumulated in the gill area and subsequently in the stomach and gut region (Fig. 1). Figure 5 displays the curves from the measurements of radioactivity in the stomach from the two mussels given bacteria (S:80 and S:800, respectively). The accumulation of S:80 in the stomach region was 11 ± 1.4% of the given amount of radioactivity and the process was slow (0.04 ± 0.01% min^{-1}) and nondynamic. Of the given activity of S:800, 32.7 ± 0.28% was measured in the stomach. The accumulation was faster (0.36 ± 0.20% min^{-1}), and the reduction came in pulses.

Figure 6 displays the curves from the measurements of radioactivity in the stomach from the two mussels fed on the colloidal suspensions (Tc+80 and Tc+800, respectively). The radioactivity from Tc+80 was 21.2 ± 3.6%, and the uptake rate was 0.14 ± 0.01% min^{-1}. For Tc+800, the uptake was 31.1 ± 2.2% and the process was faster (0.31 ± 0.02% min^{-1}). The dynamic movements of the radioactivity in the stomach were similar between these mussels. The radioactivity from the two mussels given free Tc was below the limit of detection.

DISCUSSION

S. typhimurium 395 MR10, used in this study, is a deep rough (chemotype Rd) mutant (Edebo et al. 1980), its surface is more hydrophobic and negatively charged than in most other *Salmonella*, and it forms a homogenous single-cell suspension in water. Labeling with $^{99}Tc^{m}$ using the high amount of SnF_2 (S:800) reduced the net negative charge of the bacteria as studied by use of electrophoresis. When less SnF_2 (S:80) was used, no effect on charge was discerned. Wang et al. (1995) and Allison et al. (1998) have suggested that the cell surface properties of particles will influence the preingestive selection on the labial palps. Our results are in accordance with these suggestions. The reduced net negative charge of S:800 was probably a consequence of accumulation of

TABLE 1.

One-way ANOVA on ranks (Kruskal-Wallis) comparing the electrophoretic mobility ($m^2 \times V^{-1}s^{-1}$) for *S. typhimurium*, treated with 80 and 800 µg SnF_2, respectively (S:80, S:800), and untreated *S. typhimurium* (control) (post hoc Student-Newman-Keuls).

Group	*n*	Median	25%	75%
Control	10	4.7×10^{-9}	4.1×10^{-9}	5.1×10^{-9}
S:80	10	5.4×10^{-9}	4.7×10^{-9}	6.5×10^{-9}
S:800	10	2.3×10^{-9}	2.1×10^{-9}	2.5×10^{-9}

Comparison	Difference of Ranks	p	q	P < 0.05
S:800 vs. S:80	156	3	5.6	Yes
S:80 vs. control	24	2	1.28	No
Control vs. S:800	132	2	7.06	Yes

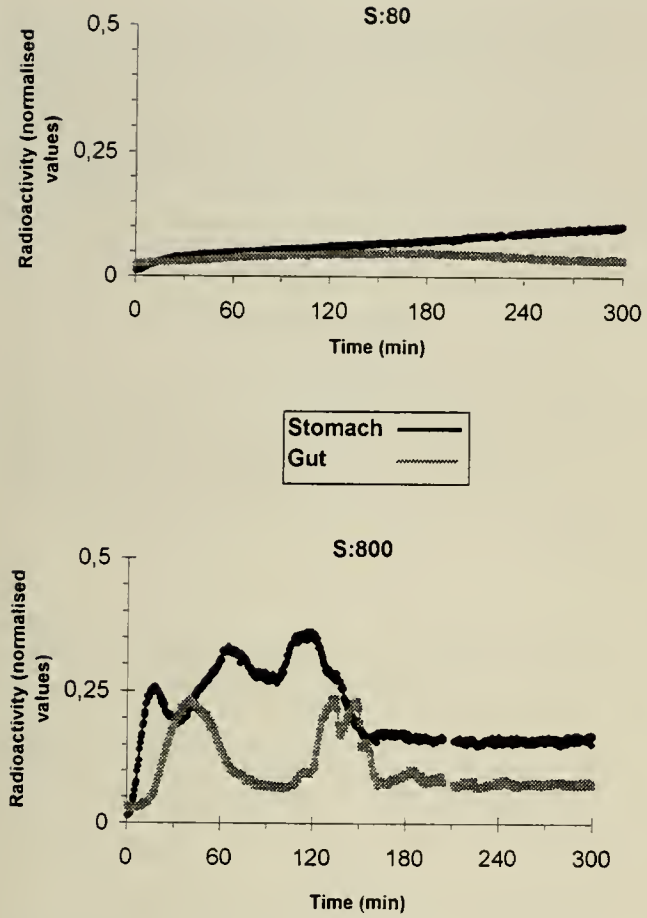

Figure 5. Curves showing the $^{99}Tc^{m}$ activity in the stomach and gut (observation time 5 h) of mussels fed *S. typhimurium*, labeled in the presence of 80 (S:80) and 800 (S:800) µg SnF_2.

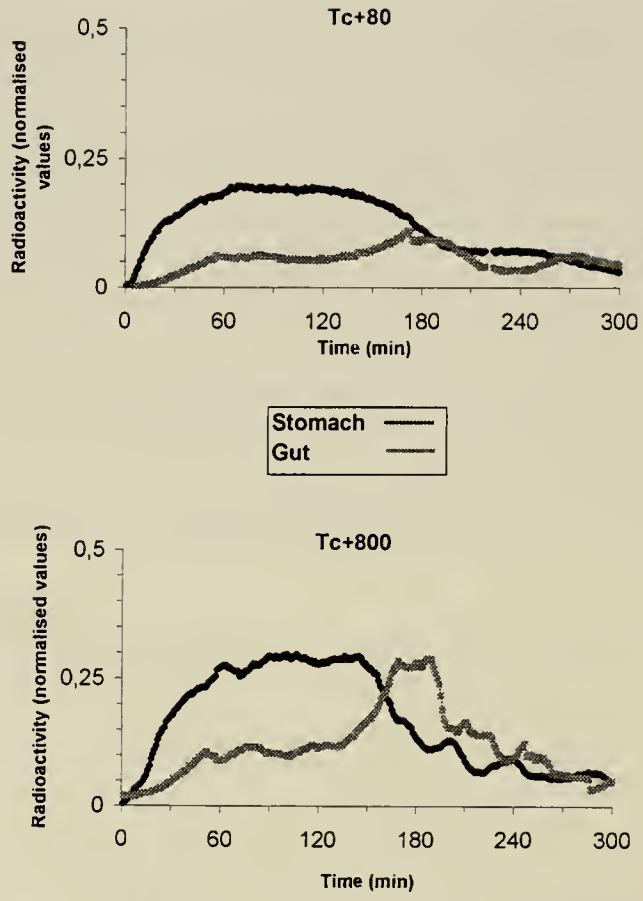

Figure 6. Curves showing the $^{99}Tc^{m}$ activity in the stomach and gut of mussels (observation time 5 h) given a suspension of by-products (probable $^{99}Tc^{m}$-stannous colloids) formed during incubation with the isotope and 80 µg SnF_2 (Tc+80) and the isotope and 800 (Tc+800) µg SnF_2.

positively charged metal complexes at its surface. These complexes might work as ligands for binding to mussel receptors or mainly operate by reducing the electrostatic repulsion between the bacterial particles and the recipient mussel surface. The differences in the cell surface properties between S:80 and S:800 might be a possible explanation for the differences shown in uptake and kinetic handling of the bacteria in the mussels.

Previous studies by Mayhew and Brown (1981) and Tseng and Wolff (1991) showed that SnF_2 inhibits the growth of the bacteria. This was also the case in our study. The viability in terms of viable counts was significantly reduced for S:80 and still more so for S:800. Bacteria in the prestationary phase were used for the labeling experiment, but log phase might have been a better condition for preserving the viability. However, the suppressed growth on agar did not correspond to the viability estimated by use of a fluorescent compound, probing the integrity of the barrier of the cell membrane, indicating that the labeling process may impair growth and division without conspicuously disturbing the cell membrane barrier. S:80 was better maintained during the incubation in seawater, but initially the viability of S:80 and S:800 was similar. Our evaluation is that the differences shown for the uptake should not be explained by differences in viability, since this phenomenon appeared directly from start when the viability of S:80 and S:800 was still comparable. The similarity between S:80 and S:800 in cell membrane integrity, size, and shape made us judge them as equal prey when given to the mussels.

According to Ross et al. (1984), the size of Tc-stannous colloids is approximately 1.5 µm, which is close to that of *S. typhimurium*. When giving the mussels the suspension with complexes formed between $^{99}Tc^{m}$ and SnF_2, without involvement of biological material, radioactivity was also accumulated in the mussels, in a way very similar to that of the more heavily labeled bacteria. These results indicate that the metal complexes on the surface of bacteria play a mediating role in the uptake process and that by-products formed when labeling the bacteria can influence the imaging analysis. The fraction of activity not bound to the bacteria was not greater than the fraction of by-products found when analyzing free pertechnetate, which indicates that it might include free or hydrolyzed pertechnetate and not only colloids. However, these fractions of "probable colloids" produced less than 4% of the total amount of radioactivity, and the influence on image analysis was considered to be of minor importance for the purpose of this study.

The amount of free or hydrolyzed pertechnetate in the bacterial suspension could not be established. The labeling efficiency was not significantly different comparing S:80 and S:800. The mean efficiency was 73.3%, and there is no evidence that the excess of $^{99}Tc^{m}$ was separated from the bacteria through the washing steps. In addition, a released fraction of radioactivity from the bacteria suspension appeared during the incubation in seawater. However, this study showed that even though the mussels were offered only

free pertechnetate (free Tc), the uptake was not detectable and did not affect the measurements. The possibility of passive diffusion of free pertechnetate cannot be excluded and needs further investigation.

The appearance of soluble $^{99}Tc^{m}$ was not significantly greater for S:800 than for S:80 after 180 min of incubation, even though the viability was more reduced. Thus, only a limited proportion of the bacteria were lysed, or lysed bacteria did not release the radiotracer. The feeding activity of the mussels can be stimulated both for particulate or nonparticulate food (Thompson and Bayne 1972). Cell leakage due to lysed bacteria could elicit a chemosensory response, which might explain the preferential uptake of S:800. However, the intact state of cell membranes and similar uptake of Tc-stannous colloids argue against such an effect. Aggregation of the bacteria would also affect the ingestion, but as no aggregates were found by the microscopic inspection, this explanation is rejected.

In summary, this study showed that there seems to be a higher uptake capacity and a more dynamic processing of the bacteria in the digestive gland due to the amount of SnF_2 used in the labeling process. The disturbance of the processing stresses the importance to keep the bacteria in a state as natural as possible. Although stannous fluoride is a toxic component to bacteria, it can be used as a reducing agent in the labeling process to produce a stable gamma-emitting bacterial tracer. However, the concentration used for this purpose has to be taken in consideration when studying uptake of viable bacteria in mussels. Bacteria labeled with gamma-emitting radionuclides, such as $^{99}Tc^{m}$-pertechnetate, have the potential to be used in numerous applications of bivalve research.

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ISOLATION AND CHARACTERISATION OF A cDNA ENCODING AN ACTIN PROTEIN FROM THE MUSSEL, *MYTILUS GALLOPROVINCIALIS*

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ABSTRACT A full-length complementary DNA encoding an actin was isolated from a *Mytilus galloprovincialis* hemocyte library. This actin displays a typical 376 amino acid open reading frame. Northern blotting indicated that the expression of the actin gene is particularly abundant in muscular tissues. This actin cDNA will be useful as a potential genetic marker as a standard for expression level in genetic regulation studies and will allow screening for the whole gene as well as its upstream regulation sequences.

KEY WORDS: Actin, *Mytilus galloprovincialis*, mollusk

INTRODUCTION

Actins are highly conserved contractile proteins ubiquitous in all eukaryotic cells. In muscle cells it is important in myofibrillar contraction, and in non-muscular cells these proteins play a role in diverse functions such as motility, phagocytosis, chromosome movements, and transport of macromolecules within the cells (Korn 1978). Muscle-specific actins can be distinguished from cytoplasmic actin in vertebrates by their primary sequences (Vandekerckhove and Weber 1978). For example, the amino acid Val 10 is characteristic of cytoplasmic actin, while Val 17 is typical of muscular actin. Wesseling proposed 3 boxes in the N-terminal region as diagnostic for the family to which an actin belongs (Wesseling et al. 1988). In invertebrates, actins also have both muscular and non-muscular functions, but these two classes are not readily distinguished on the basis of amino acid sequence. Indeed, invertebrate muscular forms of actins are closer to β-cytoplasmic pattern of vertebrate. In practice, rigorous analysis of tissue expression is necessary in order to distinguish between the different forms. Actin genes are very abundant and constitutively expressed. As such they have been subject to numerous studies also among invertebrates (Gomez-Chiarri et al. 1994; Horard et al. 1994; Lardans et al. 1997; Cadoret et al. 1999).

The bivalves rely on an innate immune defence based on both cellular and humoral components which interplay to eliminate potentially infectious microorganisms. One such innate immune mechanism is the production of antimicrobial peptides which have been recently identified in mussels of the genus *Mytilus sp.* (Hubert et al. 1996; Charlet et al. 1996; Mitta et al. 1999a,b). A deeper knowledge of this defence system would allow the establishment of health controls to detect bivalve immunodeficiency, the selection for disease resistance with a coupling of immunology and genetics or by referring to classical genetics, the characterisation of immune genes could be exploited in genetic quantitative selection. Finally, genetic transformation constitutes another promising strategy to obtain resistant strains by various modifications systems. As part of this strategy, the identification of constitutive genes like the actin, that provide tools in the study of regulation mechanism of the identified peptides was undertaken. We isolated a full-length actin cDNA and carried out initial mapping of its expression by Northern blot experiments. This is a first step toward the identification of promoter regions as well as the sequencing of the whole gene.

MATERIAL AND METHODS

Animals and Hemolymph Collection

Adult mussels (*Mytilus galloprovincialis*) were obtained from a commercial shellfish farm (Palavas, France, Gulf of Lion) during winter. The hemolymph of 20 mussels (approximately 0.5 mL/animal) was extracted via a 23G needle plus syringe, directly into an equal volume of the anti-aggregant buffer, Modified Alsever Solution (MAS, Bachère et al. 1988), and immediately centrifuged at 800 g for 15 min at 4 °C. The cell pellet was air-dried and stored at −80 °C until required.

Actin-Specific and Ribosomal 18S-Specific DNA Probes and Screening of cDNA Library

Poly(A)$^+$ RNA from adult mussel hemocytes were used to construct a cDNA library in the ZAP Express Vector (Stratagene, La Jolla). Reverse transcription and polymerase chain reaction (PCR) were used to prepare a DNA probe corresponding to hemocytic actin. Three μg of total RNA (see below for RNA isolation) were submitted to reverse transcription using the Ready-to-Go You-prime first strand beads kit (Pharmacia). One-fifth of the reaction was directly used as a template for PCR with two primers designed from a consensus actin sequence by M. E. Unger and G. Roesijadi (1993) for the oyster *Crassostrea virginica*, and renamed Avi1 (5′TAA TCC ACA TCT GCT GGA AGG TGG 3′) and Avi2 (5′TCA CCA ACT GGG ATG ACA TGG 3′). PCR was performed in 50 μL with 40 cycles consisting of 1 min at 94 °C, 1 min at 60 °C and 1 min at 72 °C with 1.5 mM $MgCl_2$ and 1 μM primers.

The resulting 846 base pair fragment corresponding to an actin cDNA fragment was cloned using the pCR-Script Amp SK (+) Cloning Kit (Stratagene, La Jolla). The plasmid containing the actin cDNA fragment was called pBSAct.846. The pBSAct.846 insert was labeled with [^{32}P] by random priming using the Ready-

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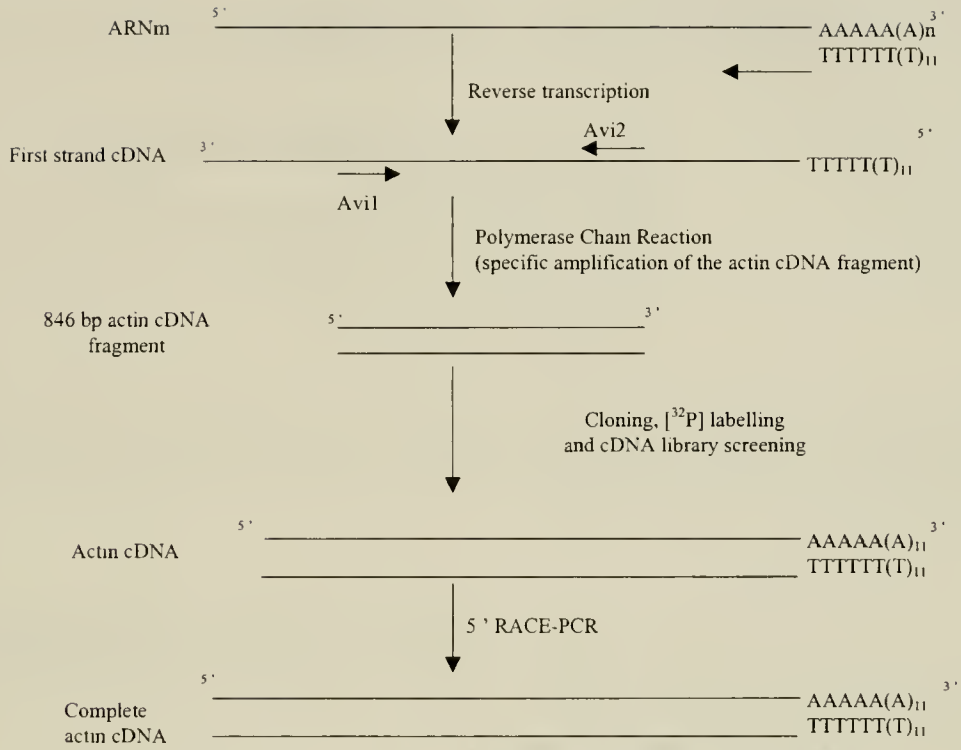

Figure 1. Complete characterization of *Mytilus galloprovincialis actin* cDNA (Mya2).

to-go DNA labeling kit (Pharmacia Biotech.) and used to screen 400,000 plaques from the cDNA library that has been transferred to Hybond-N filter membranes (Amersham Corp.). High stringency hybridization was carried out overnight at 65 °C in 5X Denhardt's solution, 5X SSPE (Sambrook et al. 1989), 0.1% SDS, 100 μg/mL salmon sperm DNA. The filters were washed in a solution of 0.5X SSC containing 0.1% SDS at 65 °C, followed by autoradiography. A secondary screening was performed to purify the positive clones. Phagemids were obtained by *in vivo* excision according to the manufacturer's instructions and sequenced on both strands.

To compare the relative expression of actin messenger in various tissues of the mussel (see Northern-blot analysis), a probe detecting a 18s rRNA, present at the same level in all tissues was designed. As such, a sense oligonucleotide primer (5'TGACCTCGCGGAAAGAGCGC 3') and an antisense oligonucleotide primer (5'AGGGGACGTAATCAACGCGAGC 3') were designed from the sequence of the ribosomal RNA small subunit (Kenchington et al. 1995) and used in PCR experiments. Five hundred ng of mussel genomic DNA were submitted to amplification in 50 μL using 35 cycles consisting of 1 min at 94 °C, 1 min at 60 °C and 1 min at 72 °C with 1.5 mM $MgCl_2$ and 1 μM primers (Fig. 1).

Northern Blot Analysis

The hemocytes from 4 mussels collected together during winter (8×10^6 cells per animal) were centrifuged and resuspended in 1 mL of Trizol (Life Technologies). Immediately after hemolymph collection, the mantle, foot, labial palps, gills, hepatopancreas, and adductor muscle were excised from the same animals and washed extensively in sterile-filtered seawater. The tissues (100 mg of each) were homogenized in 1 mL of Trizol with 30 strokes of a Potter homogenizer to break the cells in 1 mL of Trizol and total RNA was extracted according to the manufacturer's protocol (Life Technologies). Five μg of total RNA was isolated from each tissue, pooled from each animal (20 μg total per tissue) and subsequently analyzed.

Total RNA and size markers were electrophoretically separated on a 1.2% agarose gel containing 17% formaldehyde, transferred and cross-linked to a Hybond-N filter membrane (Amersham) which was then stained with methylene blue. The membrane was hybridized with the [^{32}P]-labeled actin cDNA probe in a solution containing formamide (50%), 5X SSC, 8X Denhardt's solution, sodium phosphate (0.05 M pH 6.5), SDS (0.1%) and salmon sperm (100 μg/mL) at 55 °C for 12 h. The membrane was washed in 0.2 X SSC, 0.1% SDS at 65 °C and autoradiography was carried out. After autoradiography, the membrane was stripped by incubating the blot with a boiling solution of 0.1% SDS for 1 hour and submitted to a subsequent hybridization with the [^{32}P]-labeled DNA probe revealing 18S rRNA.

Rapid Amplification of 5' cDNA end (RACE-PCR) and PCR

To obtain the complete cDNA sequence corresponding to the actin mRNA, a 5' RACE-PCR was undertaken. This was performed using the 5' RACE Kit (Boehringer Mannheim) following the manufacturer's instruction. Briefly, 2 μg of total RNA from the pooled hemocytes were submitted to reverse transcription using antisense 24 nucleotides primer (5'ATGATGTCTGTTTTATAAAGTTAT 3'), deduced from the actin cDNA sequence. After first-strand cDNA synthesis and addition of a poly(A) tail at its 5' end, PCR was performed with an oligo d(T)-anchor primer and a nested antisense primer of 24 nucleotides (5'AGAGGAGTATCTCACCCTGACTTC 3') deduced from the actin cDNA sequence. Amplification was performed according to the following program: melting at 94 °C for 1 min, annealing at 50 °C for 1 min, elongation at 72 °C for 1 min (35 cycles). The PCR products were cloned using the pCR-Script Amp SK (+) Cloning Kit (Stratagene) and several different cDNA clones were sequenced.

```
   1  ttttttacca gtttgttgta gaagtcaggg tgagatactc ctctttagcg
                                      M   C  D  D   K  V  A          7
  51  tttagtataa ctttataaaa cagacatcAT GTGTGACGAC AAAGTAGCCG
      A  L  V  V   D  N  G   S  G  M  C   K  A  G   F  A  G         24
 101  CTTTGGTAGT AGACAATGGA TCAGGAATGT GCAAAGCTGG TTTCGCCGGA
       N  D  A  P   R  A  V   F  P  S   I  V  G  R   P  R  H       41
 151  AATGATGCTC CAAGAGCCGT GTTTCCCTCC ATCGTTGGAA GACCAAGACA
         Q  G  V   M  V  G  M   G  Q  K   D  S  Y   V  G  D       57
 201  TCAGGGAGTC ATGGTTGGTA TGGGTCAGAA AGACTCCTAC GTAGGAGATG
      E  A  Q  S   K  R  G   I  L  T  L   K  Y  P   I  E  H       74
 251  AAGCCCAGAG CAAGAGAGGT ATCCTCACCC TGAAATACCC AATTGAGCAC
       G  I  V  T   N  W  D   D  M  E   K  I  W  H   H  T  F      91
 301  GGTATCGTCA CAAACTGGGA CGATATGGAA AAAATCTGGC ATCACACCTT
         Y  N  E   L  R  V  A   P  E  E   H  P  V   L  L  T      107
 351  CTACAACGAA CTCCGTGTTG CCCCAGAAGA GCACCCAGTC CTTCTGACTG
      E  A  P  L   N  P  K   A  N  R  E   K  M  T   Q  I  M      124
 401  AGGCTCCACT CAATCCCAAA GCCAACAGGG AAAAGATGAC CCAGATCATG
       F  E  T  F   N  A  P   A  M  Y   V  A  I  Q   A  V  L      141
 451  TTCGAGACCT TCAATGCACC AGCCATGTAC GTCGCTATCC AGGCCGTACT
         S  L  Y   A  S  G  R   T  T  G   I  V  L   D  S  G      157
 501  CTCACTGTAT GCTTCCGGTC GTACCACTGG TATCGTACTC GACTCTGGAG
      D  G  V  T   H  T  V   P  I  Y  E   G  Y  A   L  P  H      174
 551  ATGGTGTCAC ACACACCGTA CCAATCTACG AAGGTTACGC TCTTCCCCAC
       A  I  L  C   L  D  L   A  G  R   D  L  S  D   N  W  M      191
 601  GCCATCCTCT GTCTAGACTT GGCCGGTAGA GATCTTAGTG ATAACTGGAT
         K  I  L   T  E  R  G   Y  S  F   T  T  T   A  E  R      207
 651  GAAAATCCTC ACCGAGAGAG GTTACTCATT CACAACCACC GCGGAGAGAG
      E  I  V  R   D  I  K   E  K  L  C   Y  V  A   L  D  F      224
 701  AAATCGTTAG AGACATTAAG GAAAAATTGT GCTATGTTGC TCTTGATTTC
       E  Q  E  M   S  T  A   A  S  S   S  S  L  E   K  S  Y      241
 751  GAGCAGGAAA TGTCAACCGC CGCTTCTTCA TCTTCCCTAG AAAAGAGCTA
         E  L  P   D  G  Q  V   I  T  I   G  N  E   R  F  R      257
 801  CGAATTGCCC GATGGACAGG TTATCACCAT TGGTAACGAA AGATTCAGGT
      C  P  E  S   L  F  Q   P  S  F  L   G  M  E   S  A  G      274
 851  GTCCAGAATC ATTATTCCAA CCATCCTTCT TGGGTATGGA ATCTGCTGGT
       I  H  E  T   T  Y  N   S  I  M   K  C  D  V   D  I  R      291
 901  ATCCATGAAA CCACATACAA CAGTATCATG AAGTGTGATG TCGATATCCG
         K  D  L   Y  A  N  T   V  L  S   G  G  T   T  M  F      307
 951  TAAGGACTTG TACGCCAACA CCGTCTTGTC TGGTGGTACC ACCATGTTCC
      P  G  I  A   D  R  M   Q  K  E  I   T  A  L   A  P  S      324
1001  CAGGTATTGC CGACAGAATG CAGAAGGAAA TCACAGCACT TGCTCCAAGC
       T  M  K  I   K  I  I   A  P  P   E  R  K  Y   S  V  W      341
1051  ACAATGAAGA TCAAAATCAT TGCCCCACCA GAGAGGAAAT ACTCCGTCTG
         I  G  G   S  I  L  A   S  L  S   T  F  Q   Q  M  W      357
1101  GATCGGTGGT TCCATCTTGG CTTCATTGTC CACCTTCCAA CAGATGTGGA
      I  S  K  Q   E  Y  D   E  S  G  P   S  I  V   H  R  K      374
1151  TCAGCAAACA GGAATATGAC GAATCTGGCC CATCCATTGT CCACAGGAAA
       C  F  *                                                    376
1201  TGCTTCTAAa ctaaattgtt ttctaggact tatattaatt tattttcaaa
1251  tttcgttaaa acaaaaagtt tcgtgcttgg taacatggac tttaatttat
1301  acaaactgtc tttaaccctt tcaaacttca gatctgtatt agcattgagc
1351  taacggtact tgtacaaata taggacagta aattattatt tgttttatgt
1401  gaaaaagtct ggtggttcaa atgcaagaat gtggagagtt gaatgtgaaa
1451  aagacttgta aaaatactaa acaatccgga aacatatttc aggtttccag
1501  gggagataac tttttactaa atttgatgta catgtggaat aaatcatctg
1551  cattattgtg ataaaatgac ctttatacat ccaattatat taaatcttat
1601  aaaaaaaaaa aaaaaaaa
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Figure 2. Nucleotide sequence and deduced amino acid sequence of the *Mytilus galloprovincialis actin* cDNA (Mya2). Untranslated regions in lower-case letters. Start codon in boldface letters. Polyadenylation signal is underlined.

RESULTS AND DISCUSSION

After colony blot of the cDNA library, 5 positive clones among 352 were chosen and submitted to secondary screening for isolation. The corresponding phagemids were obtained by *in vivo* excision and the longest was sequenced on both strands (Fig. 2). This complete actin cDNA (named *Mya2*, Genbank accession number AF157491) shows a potential coding sequence stretching over 1618 bp and codes for a typical 376 amino acids actin. The 5′ RACE-PCR experiment allowed an additional 19 base pairs to be added and helped to suggest the Transcription Start Point (+1). Best homologies in amino acid sequence were found with the bivalve *Placopecten magellanicus;* 97.8%, the nematode *Caenorhabditis elegans;* 96.2%, the brine shrimp *Artemia sp;* 96.5% and the silk worm *Bombyx mori;* 96.2%. For nucleic acid sequence, best homologies are found with the scallop *Placopecten*

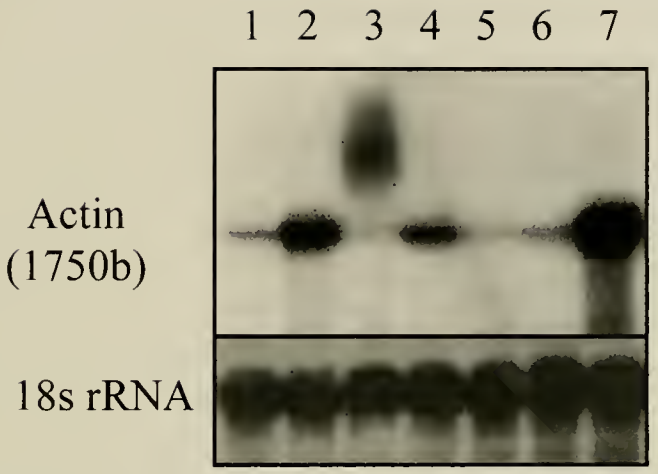

Figure 3. Northern blot analysis of RNAs from various tissues of the mussel. Twenty µg of total RNAs from various tissues: 1, hemocytes; 2, mantle; 3, foot; 4, labial palps; 5, gills; 6, hepatopancreas; 7, adductor muscle. All were separated by 1% agarose-formaldehyde gel electrophoresis, blotted and hybridized with ^{32}P-labeled cDNA probe corresponding to actin cDNA. The RNA relative amounts of the various tissues are evaluated by hybridizing the same membrane with a probe corresponding to the 18S rRNAs because the actin mRNA probed is differentially expressed in the different tissues tested.

magellanicus (85%), the zebra mussel *Dreissena polymorpha* (84%) and the oyster *Crassostrea gigas* (83%). According to Vandekerckhove and Weber, (1978) who described 20 residues discriminating muscular and cytoplasmic actins, *Mya2* displays feature of cytoplasmic actin for 10 codons, while 3 of them show the mark of muscular actins. The cystein in position #2 is a common feature among invertebrate actins, although some exceptions are documented. Actin mRNAs were detected in various tissues as demonstrated by Northern Blot experiments using the *Mya2* cDNA as probe (Fig. 3). The signal is particularly strong in the mantle, the labial palps, and the adductor muscle. This strong signals, however, are mainly due to the recognition by the probe of all forms of actin mRNA. Indeed, conservation is so high (particularly within the used probe) that both muscular and cytoplasmic forms are highlighted giving a cumulative signal.

Several isoforms have been reported in higher vertebrates, divided into muscular and non-muscular actins (Rubenstein 1990). Due to the high level of similarity with the other actin genes, this sequence may not be suitable for intra- and inter-species phylogenetic studies. Nevertheless, the potential availability of intronic non-expressed sequences within this actin gene would be of interest in developing a selectively neutral marker as has already been done in other bivalves (Corte-Real et al. 1994; Ohresser et al. 1997). Furthermore, this complete cDNA sequence can now be used in regulation studies as an expression level standard as well as an anchor in the search for the complete gene including the proximal promoter involved in its expression pattern.

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GROWTH OF SEED MUSSEL (*MYTILUS GALLOPROVINCIALIS* LMK): EFFECTS OF ENVIRONMENTAL PARAMETERS AND SEED ORIGIN

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ABSTRACT Mussel seeds (*Mytilus galloprovincialis* Lmk) of similar weight and length from two different origins (rocky shore and collector ropes) were cultivated on a raft in the Ría de Arousa (northwest Spain), from seeding to thinning out, for a total period of 208 days (November 1995 through July 1996). Weight increase rates for the seed from collector ropes were higher than those for the seed from rocky shore, and the growth rate variations during the cultivation period were associated with the environmental parameters measured (chlorophyll *a* and temperature). The origin of the seed was also found to be a significant factor. The condition index (CI) of the seed from collector ropes was significantly greater than that of the rocky shore seed at the beginning of the cultivation time. Both mussel seeds showed a similar CI after 70 days and during the rest of the cultivation time. Although allometric coefficient values for the relation total dry weight-length showed a similar range for both types of seed, no significant differences were observed for this coefficient in collector rope mussels throughout the cultivation period. Rocky shore mussels showed, on the contrary, a significant increase for this allometric coefficient value throughout the cultivation period. These preliminary results from the total dry weight-length relationship obtained here and the change of CI differences serve to strengthen the hypothesis of a physiological basis for the differences in growth between both types of seed mussel. This finding could be related to the different features of the original habitats of the two types of seed, in terms of the cycles of availability of food and exposure to the air.

KEY WORDS: Mussel, growth, environmental parameters, condition index, allometric functions

INTRODUCTION

Mussel (*Mytilus galloprovincialis* Lmk) cultivation in Galicia and other cultivation zones (Pérez Camacho et al. 1995) is dependent on the availability of large quantities of seed, which can be obtained from two very different origins: coastal stocks from the rocky shoreline, and collector ropes suspended from cultivation rafts.

Previous studies about the growth of these two types of seed in the Ría de Arousa disagree as to their growth potential from seeding to thinning out. On the one hand, Pérez Camacho et al. (1995) found differences in growth rates and condition indices of the mussels that they attributed to the origin of the seed, with collector rope seed having the highest values. On the other hand, Fuentes et al. (1998) concluded that neither of the two types of mussel seed (rocky shore and collector rope) has a higher growth potential, although the authors do recommend that mussel farmers "use seed from collector ropes due to their significantly larger size at harvest time."

Dickie et al. (1984), Page and Hubbard (1987), and Fuentes et al. (1992) have all established that the origin of the seed has a significant effect on mortality rates, although not on growth. However, Peterson and Beal (1989) and Rawson and Hilbish (1991) have observed a significant effect of origin on growth, which they explain as being due to genetic differences.

Bayne and Newell (1983) point to the effect of endogenous factors (physiological condition, size, and genotype) and the specific environmental conditions of the area in question as being two of the factors that most affect growth in bivalve molluscs. In the case of environmental factors, it has been shown that in areas where temperature, for example, is not a limiting factor, the availability of food affects growth to a very large extent (Mallet et al. 1987, Stirling and Okumus 1994, Sukhotin and Maximovich 1994, Widdows et al. 1997).

The aim of this study was to investigate the effects of seed origin and environmental parameters on different growth indicators (growth rate, condition index [CI], and the allometric relation weight-length).

MATERIALS AND METHODS

Experimental Design

Seed of *Mytilus galloprovincialis* Lmk, approximately 20 mm long, was gathered from the rocky coastline and from raft collector ropes in the mid-to-outer area of the Ría de Arousa (Galicia, northwest Spain) in November 1995. Both types of seed, from the same year class, came from the spawning period in the previous spring-summer, and the sampling locations were 2 km away from each other. Experimental cultivation, which was carried out in a raft usually used for the culture in the Ria de Arousa (500 m^2), commenced in winter in order to minimize any possible advantages that collector rope seed may have as a result of its being better adapted to cultivation on the raft. The experiment ran until June 1996 (208 days), thus covering the first stage in mussel cultivation, from seeding to thinning out (50–60 mm). Sixteen cultivation ropes (12 m) were used, eight for each type of seed, alternately placed and having a density of 19 kg of seed per rope (1.6 kg/m of rope or 2,600 individuals per meter of rope). Sampling was performed by removing mussels from adjacent ropes at an average depth of 2–4 m for both types of seed.

Initial average lengths (± standard deviation) were 22.5 ± 1.5 mm for the seed from collector ropes and 19.0 ± 1.9 mm for that from the rocky shore. Average total dry weights were 0.36 ± 0.06 and 0.27 ± 0.06 g/individual, respectively. No significant differences were observed for length and dry weight between both types of seed at the outset of the experiment (analysis of variance [ANOVA]; $P > 0.05$).

Environmental Parameters

Natural seston was described as total particulate matter (TPM, mg/L), particulate organic matter (POM, mg/L), particulate inor-

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ganic matter (PIM, mg/L), total particulate volume (Vol, mm^3/L), and chlorophyll *a* (chl-*a*, µg/L). The quality of the seston was expressed as Q_1 (POM/TPM) and by the chl-*a*/TPM index.

The values of chl-*a*, as well as temperature (°C) and salinity (‰) of the water column, were supplied by the Marine Environment Quality Control Centre of the Consellería de Pesca, Marisqueo e Acuicultura (Ministry of Fisheries, Shellfisheries and Aquaculture) of the Xunta de Galicia (Galician Regional Government). chl-*a* was calculated from the fluorescence data.

Seawater samples were filtered onto pre-ashed (450°C for 4 h) and weighed GFC filters and rinsed with isotonic ammonium formate (0.5 M). Total dry matter was established and the weight increment determined after drying the filters to constant weight at 110 °C for 12 h with an accuracy of 0.001 mg. Organic matter corresponded to the weight loss after ignition at 450 °C for 4 h in a muffle furnace. Particulate volume per liter of seawater was determined by counting in the range of 2–56 µm using a Coulter Counter Multisizer II fitted with a 100 µm-aperture tube.

Mussel Sampling

Duplicate samples of 200–350 individuals were taken from adjacent ropes, which corresponded to both types of seed mussel after 70, 148, and 208 days.

Individual mussel length (L) was measured to the nearest 1 mm using calipers, and each sample was divided into 1-mm length classes. Adjusted length was given by the formula: $L = (C_L\ F)/N$ (Box et al. 1989), where C_L is the individual length class, F is the frequency, and N is the total number of individuals. Subsamples of 5–15 mussels were each taken from five to six length classes covering the entire size range and used to determine total dry weight (DW_{total}) and organic weight of tissues (OW_{tissue}). After cutting adductor muscles and allowing intervalvar water to drain by placing the mussels with their ventral edge on filter paper, tissues were dissected and both shell valves (DW_{shell}) and soft tissues (DW_{tissue}) were weighed after drying at 100°C until constant weight was obtained. We ashed the soft tissues at 450°C for 48 h to determine OW_{tissue}, with an accuracy of 0.01 g in all cases.

CI was calculated from the ratio of tissue dry weight (DW_{tissue}) and the dry weight of the valves (DW_{shell}) according to the equation $CI = (DW_{tissue} / DW_{shell})\ 100$ (Freeman 1974).

Data Analysis

Regression models were calculated for the logarithm of total dry weight (log DW_{total}), tissue dry weight (log DW_{tissue}), and tissue organic weight (log OW_{tissue}) versus logarithm of length (log L) relationships from data obtained for five or six length classes covering the entire length class range from 10–15 mm above and below the mean length: $\log W = \log a + b \log L$. Analysis of covariance (ANCOVA; Snedecor and Cochran 1980) was used to make a comparison of these functions between both types of seed mussel and the change of allometric coefficient (b) in the experiment.

The confidence interval for the difference in length and weight between the months of the cultivation period studied that gives the growth rate for each stage was given by the formula: $X_{t+1} - X_t \pm [t_{(1-\alpha/2,k)}\ Sp \sqrt{(1/n_{t+1} + 1/n_t)}]$ (Canavos 1988), where X_{t+1} and X_t are the mean values for length and weight at each end of the intervals, Sp^2 is the variance at each end of the interval, n_{t+1} and n_t are the number of samples at each end of the interval, and $t_{(1-\alpha/2,k)}$ is the Student *t*-distribution value with 95% confidence and k degrees of freedom ($k = n_{t+1} + n_t - 2$).

Comparison of mean values of growth rate was carried out with an ANOVA. Homogeneity of variances was tested by the Bartlett test (Snedecor and Cochran 1980), and correction for heterogeneity (when required) was performed by reciprocal or logarithmic transformation data. In cases in which homogeneity was not obtained after these transformations had been carried out, the Kruskall-Wallis nonparametric test was used.

The effects of environmental parameters and origin of seed mussel on the growth rate were tested by stepwise multiple regression. Seed origin was introduced with values 0 and 1 for collector rope and rocky shore mussels, respectively. Length and dry weight values of growth rate were transformed by $\log_{10}(x + 1)$ to stabilize variances.

RESULTS

Environmental Parameters

Variation in temperature (°C) took place within a narrow range, there being a difference of only 2.7 °C between the maximum and minimum temperatures during the whole of the experimental period (Fig. 1A). Temperature was high at the beginning of the cultivation period (15.5 °C) and then decreased in zigzag until the minimum temperature was reached in February (12.9 °C). From then on, throughout the spring months, there was a steady increase in temperature.

Salinity (‰) was dependent on rainfall. Average values for the area (31.3–35.2‰) were obtained at the outset, and they gradually decreased until January, when the minimum value (28.0‰) was recorded. Salinity then increased during the spring months and finally reached its maximum value at the end of the cultivation period in July (35.6‰; Fig. 1A).

High values for TPM were registered in February through April (0.9–1.4 mg/L; Fig. 1B), in contrast with the low values obtained throughout the winter months. However, the maximum of TPM occurred at the beginning of January (2.6 mg/L; Fig. 1B), which can be related to maximums in POM (1 mg/L) and especially in PIM (1.6 mg/L). With the single exception of this maximum value,

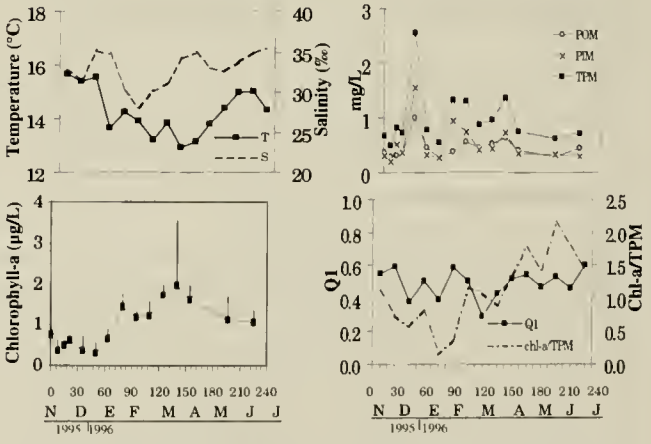

Figure 1. Variation of average values (mean SD) of temperature (°C) and salinity (‰) (A); TPM (mg/L), POM (mg/L), and PIM (mg/L) (B); chl-*a* (g/L) (C); and quality of the seston (Q_1 µg POM/TPM) and chl-*a*/TPM index (D), during the experimental period November 1995 through July 1996.

POM was higher during the spring (>0.5 mg/L) than during the winter (0.3 mg/L). Fluctuations in chl-*a* produced two peaks in February and April (1.4 and 2.0 μg/L, respectively; Fig. 1C) after the low values recorded during the initial stages of the experiment (0.3–0.8 μg/L).

Q_1 varied between 0.3 and 0.6, showing a greater oscillation in winter and a narrower range of fluctuation around 0.5 during spring, which corresponds to the value that is generally obtained for the Ría de Arousa (Fig. 1D). The chl-*a*/TPM index varies to a much greater extent, with low values being recorded in winter (0.1–1.1; Fig. 1D) and then increasing from February on to reach a peak in April and June (2.1).

Growth

The growth rate in terms of length (mm/mo) shows minimum values in winter (1.5 and 2.0 mm/mo for collector rope and rocky shore mussels, respectively; $P > 0.05$) and maximum levels from April through June (9.1 and 6.8 mm/mo for the same two mussel populations, respectively; $P > 0.05$; Table 1). The average growth rates for the whole period November through June were thus similar for both types of mussel seed, at 4.8 and 4.5 mm/mo, respectively ($P > 0.05$).

Weight growth shows a trend similar to that for length over the cultivation period, with the minimum in winter (0.07 g DW_{total}/mo for both seed types) and the maximum in the April through June period, when the collector rope mussels showed significantly higher values (1.60 g DW_{total}/mo) than the rocky shore mussels (0.86 g DW_{total}/mo) ($P < 0.05$; Table 1). The overall November through June values for DW_{total} growth rates are 50% higher for the former (0.61 g DW_{total}/mo) than for the latter (0.41 g DW_{total}/mo) ($P < 0.05$; Table 1). The differences between these two groups of mussels in the final stages of cultivation (April through June) and in the overall average values (November through June) also apply to organic and dry weight of tissues (OW_{tissue} and DW_{tissue}, respectively), with collector rope mussels once again showing higher values (see Table 1).

The variation of growth rate in terms of both length and total dry weight in this study bore a significant relationship to fluctuations in the environmental parameters chl-*a* and temperature of the water column, in this order of importance (Table 2). Both of these environmental variables show positive and significant coefficients ($P < 0.001$ for chl-*a* and $P < 0.05$ for temperature vs. growth rate for length), with chl-*a* being the major component of the variance (40.1 and 56.6% for growth rates for dry weight and length, respectively). A significant but residual effect ($P = 0.040$) was also noted for seed origin vs. growth rate for total dry weight (Table 2).

Condition Index

CI for collector rope mussels was 33% higher than that for rocky shore mussels ($P < 0.001$) at the beginning of the cultivation period (Table 3). After 70 days, similar values of CI were obtained for both groups of mussels ($P > 0.05$), and this remained the case until the end of the cultivation period without differences between them. The significant increase in CI ($P < 0.001$) for both groups of mussels between 70 and 148 cultivation days, which corresponds with the period February through April, is remarkable.

Allometric Functions

Values a and b of the allometric function weight-length ($W = a L^b$) for each mussel seed during the cultivation period are shown in Table 4. No significant differences among the slopes (b) of both groups of mussels at any time during the cultivation period were detected when an ANCOVA was performed on the linear transformations of these functions ($P > 0.05$). However, the intercepts for collector rope mussels were significantly higher at the end of the cultivation period (June) in all cases ($P < 0.001$ for DW_{total} and OW_{tissue} vs. L and $P < 0.01$ for DW_{tissue} vs. L; Table 4). Significant differences were also obtained for the intercepts in February ($P < 0.05$) and November ($P < 0.01$) for the relations DW_{tissue} and OW_{tissue} versus L, respectively, in which higher values were once more recorded for the collector rope mussels (Table 4).

Concerning shell weight, we found no differences at the onset of the experiment (0.32 ± 0.05 and 0.25 ± 0.06 g for collector rope and rocky shore mussels, respectively; $P > 0.05$). The same tendency was maintained during the cultivation period except at the end (June), when mussels from collector ropes presented heavier shells (3.63 ± 0.17 g) than rocky shore ones (2.41 ± 0.23 g) ($P < 0.001$).

A second ANCOVA was performed on the fluctuation of the values a and b in the relation DW_{total}-L over the cultivation period, for each seed type independently. The results are shown in Table 5. The power b gives similar values throughout the cultivation period for collector rope mussels ($P > 0.05$), yet when intercept a is recalculated for a common power (Rec.a), it gradually increases over time, with significant differences between November and April ($P < 0.05$) and maximums occurring in June ($P < 0.001$). On

TABLE 1.

Growth rates of mussels from collector ropes and rocky shore in different periods of culture.

	L (mm/mo)		DW_{total} (g/mo)		DW_{tissue} (g/mo)		OW_{tissue} (g/mo)	
Period of Cultivation	Collector Ropes	Rocky Shore	Collector Ropes	Rocky Shore	Collector Ropes	Rocky Shore	Collector Ropes	Rocky Shore
Nov–Feb	1.5 ± 1.4	2.0 ± 1.3	0.07 ± 0.07	0.07 ± 0.05	$4.10^{-3} \pm 7.10^{-3}$	$6.10^{-3} \pm 5.10^{-3}$	$2.10^{-3} \pm 5.10^{-3}$	$4.10^{-3} \pm 4.10^{-3}$
Feb–Apr	4.4 ± 1.5	4.9 ± 1.3	0.35 ± 0.11	0.37 ± 0.09	0.12 ± 0.02	0.11 ± 0.02	0.10 ± 0.02	0.10 ± 0.02
Apr–Jun	9.1 ± 2.0	6.8 ± 2.1	1.60 ± 0.26*	0.86 ± 0.23	0.37 ± 0.06*	0.19 ± 0.06	0.32 ± 0.06*	0.17 ± 0.05
Nov–Jun	4.8 ± 0.45	4.5 ± 0.5	0.61 ± 0.07*	0.41 ± 0.06	0.15 ± 0.02*	0.10 ± 0.02	0.13 ± 0.02*	0.09 ± 0.01
Percentages	6%		50%		55%		52%	

Data are means (n = 5 samples) ± standard deviation. L, length; DW_{total}, total dry weight; DW_{tissue}, dry weight of soft tissues; OW_{tissue}, organic weight of soft tissues.

* Significant differences between both sources of mussels ($P < 0.05$; ANOVA). Percentage values indicate how much higher is the increment of growth parameters in collector ropes mussels over total time of culture (November through June, 208 days).

TABLE 2.

Multiple regression analysis of shell length (L) and total dry weight (DW_{total}) increment on water temperature (in °C) and chlorophyll-*a* (in µg/L).

Parameter	Constant	Chlorophyll-*a*	Temperature	Origin
A. L, mm/mo	−2.855 ± 1.131	0.527 ± 0.091† (56.6%)	0.210 ± 0.078* (70.8%)	−0.011 ± 0.008
B. DW_{total}, g/mo	−2.900 ± 0.392	0.247 ± 0.035† (40.1%)	0.197 ± 0.030† (84.1%)	−0.053 ± 0.026* (87.8%)

A. $N = 18$; $r^2 = 0.708$; $F_{2,15} = 18.155$; $P < 0.001$
B. $N = 18$; $r^2 = 0.878$; $F_{3,14} = 33.620$; $P < 0.001$

Mean intercept and coefficients ± SD. Origin is defined with values 0 and 1 for collector ropes and rocky shore mussels respectively. Percentage values mean proportion of accumulated variance with inclusion of different factors (NS not significant).
* $P < 0.05$, significant difference from 0.
† $P < 0.001$, significant difference from 0.

the other hand, rocky shore mussels showed a steady and significant increase of slope (b) over time ($P < 0.05$; Table 5), reaching maximum values in June (2.507), although significant differences were already evident between the allometric coefficients for November (2.276) and April (2.491) ($P < 0.01$).

DISCUSSION

The variations in factors such as temperature, salinity, and chl-*a* in the area studied are consistent with previous descriptions of the Galician Rías (Fraga 1996). Abundant rainfall and low levels of sunlight until February are the reason for low salinity and the concentration of chl-*a* in the winter months. The maximum values of TPM and POM that occurred in January constitute an exception to the winter-spring pattern that characterizes the natural seston variability and reveal the effect that frequent storms have on a shallow area such as this at this time of year, leading to a resuspension of previously sedimented particles. The mainly sedimentary origin of this sudden increase in POM in January is supported by the low winter values of the chl-*a*/TPM index. The peak levels of phytoplankton that occur in the Galician Rías can be related either to an increase in sunlight (the first chl-*a* peak occurs in mid-February) or to the upwelling of nitrates/silicates of the water caused by the appearance of North Atlantic Central Water (NACW). NACW is the main reason for the spring upwelling in the Galician Rías, which is represented by a second and higher chl-*a* peak in mid-April.

Among the environmental parameters studied, the availability of plankton in the water column in the form of chl-*a* and water temperature had a significant effect on the variations in growth rate. Both of these factors have previously been signalled as being responsible for most of the variation in the growth rate of bivalve molluscs (Bayne and Newell 1983), and the fact that in the present study chl-*a* has the greater effect of the two supports earlier results (Pérez Camacho et al. 1995). In temperate waters, such as Ría de Arousa, temperature fluctuations are not as marked as they are in extreme environments where fluctuations in this factor play a more important role (Kautsky 1982, Sukhotin and Kulakowski 1992). Therefore, variations in growth rate in temperate waters have been associated with the availability of food (Page and Hubbard 1987, Thompson and Nichols 1988, Fernández-Reiriz et al. 1996).

Growth rate variation, estimated here with low values during winter and maximums in spring, follow a pattern similar to that found in other studies (Freeman and Dickie 1979, Pieters et al. 1980, Kautsky 1982, Loo and Rosenberg 1983, Skidmore and Chew 1985, Page and Hubbard 1987, Mallet et al. 1987). The maximum growth rates for length, which were recorded in spring (9.1 and 6.8 mm/mo for collector rope and rocky shore mussels, respectively), agree with the findings described by Pérez Camacho et al. (1995) for the same time of year and both types of seed in the Ría de Arousa. The increase in length after the experimental period (31–33 mm for 208 days; 4.4–4.7 mm/mo, with both types of seed included) is comparable to that of a previous paper on the Ria de Arousa for a similar period of the year, 5.6–5.8 mm/mo (Fuentes et al. 1998). Pérez Camacho et al. (1995) found higher growth rates of 7–9 mm/mo. However, it is necessary take into account that this experiment began in April and ended 90 days later, which means favorable conditions from the beginning with regard to temperature and seston availability and quality. The lower growth rates obtained by Fuentes et al. (1992) also with *M. galloprovincialis* in the Ria de Arousa (2.4 mm after 3 mo) can be attributed to the cultivation technique used (plastic cages).

These differences also appear in the cultivation period needed before thinning out, which is greater in experiments that commence in winter (5 and 7 mo, respectively, for Fuentes et al. 1998 and the present study) than in those that start in spring (3 mo; Pérez Camacho et al. 1995).

Although Fuentes et al. (1998) recommend that seed from collector ropes should be used for cultivation, since it reaches greater length and/or weight than rocky shore seed, they differ from Pérez Camacho et al. (1995) as to the existence of a difference in growth rates from seeding to thinning out. Their reasoning is based on the fact that if more than one cohort were included in the process of gathering the rocky shore seed, this may well explain the different growth rates reported by the latter authors. The results of this study

TABLE 3.

Condition index (CI) values for both types of seed mussel during their cultivation on a raft.

Month	Days of Cultivation	CI	
		Collector Ropes	Rocky Shore
November	0	15.84 ± 2.44*	11.87 ± 0.97
February	70	13.27 ± 0.87 NS	12.08 ± 1.86
April	148	33.11 ± 4.10 NS	30.39 ± 2.62
June	208	30.08 ± 2.87 NS	28.88 ± 3.26

NS, not significant ($N = 12$ in all cases).
* Differences highly significant.

TABLE 4.

Results of regression and covariance analysis on data relating weight (W mg) of *M. galloprovincialis* from two sources of seed to length (L mm).

Month	Collector Ropes a	Rec. a	b	r^2	n	*a Common*	*b Common*	Rocky Shore a	Rec. a	b	r^2	n
DW_{total} versus L: A												
November	0.328 NS		2.247 ± 0.092 NS	0.986	10	0.325	2.267	0.326		2.274 ± 0.056	0.996	10
February	0.152 NS		2.505 ± 0.068 NS	0.990	15	0.171	2.464	0.194		2.430 ± 0.062	0.992	15
April	0.232 NS		2.397 ± 0.196 NS	0.943	11	0.207	2.442	0.179		2.491 ± 0.054	0.996	11
June	0.644*	0.321	2.212 ± 0.118 NS	0.978	10		2.383	0.167	0.273	2.507 ± 0.098	0.988	10
DW_{tissue} versus L: B												
November	0.019 NS		2.521 ± 0.158 NS	0.970	10	0.026	2.398	0.031		2.318 ± 0.087	0.988	10
February	0.026†	0.036	2.361 ± 0.104 NS	0.976	15		2.279	0.041	0.031	2.200 ± 0.102	0.972	15
April	0.047 NS		2.464 ± 0.161 NS	0.962	11	0.040	2.508	0.033		2.557 ± 0.123	0.980	11
June	0.043‡	0.039	2.523 ± 0.260 NS	0.922	10		2.546	0.030	0.031	2.569 ± 0.350	0.964	7
OW_{tissue} versus L: C												
November	0.012‡	0.027	2.580 ± 0.208 NS	0.951	10		2.355	0.028	0.018	2.223 ± 0.085	0.980	10
February	0.018 NS		2.385 ± 0.117 NS	0.980	15	0.017	2.380	0.016		2.374 ± 0.251	0.872	15
April	0.046 NS		2.429 ± 0.170 NS	0.958	11	0.039	2.473	0.032		2.522 ± 0.126	0.978	11
June	0.037*	0.035	2.526 ± 0.275 NS	0.914	10		2.540	0.028	0.027	2.554 ± 0.232	0.960	7

a and b values are parameters in the equation $W = aL^b$; ANCOVA ANOVA was made after logarithm transformation: log W = log a + b log L. When there were no differences in slopes (b) of the relationship, a common exponent was therefore calculated and used to recalculate values for the parameter a (Rec. a). NS, not significant.

* $P < 0.001$.

† $P < 0.05$.

‡ $P < 0.01$.

do nevertheless point out a difference in growth rates, especially for weight, that are also related to variability of environmental parameters during the cultivation period. This study is also in concordance with Pérez Camacho et al. (1995), who showed that initial size (weight/length) has no effect on the results, either because the experiment was designed with this condition in mind (as in this study) or because the statistical analysis (multivariate ANOVA) performed on the results showed this to be the case (Pérez Camacho et al. 1995). With reference to the study by Fuentes et al. (1998), the differences observed in the initial size of both types of seed (0.6 cm for rocky shore mussels and 2.1 cm for collector rope mussels), as well as the high density of mussels on the ropes (5,000 individuals per meter), which contrasts with the 2,600 individuals per meter of the present study and the 2,000 individuals per meter of Pérez Camacho et al. (1995), may have affected their results, given the effect that both of the above-mentioned factors (initial size and density) may have on growth (Sukhotin and Maximovich 1994, Eldridge et al. 1979, Fernández-Reiriz et al. 1996).

TABLE 5.

Results of ANCOVA on data relating DW_{total} (mg) to shell length (mm).

Month	Collector Ropes h	a	Rec. a	n	Rocky Shore h	a	Rec. a	n
November	2.247	0.328	0.209	10	2.276	0.326	—	10
February	2.505	0.152	0.237	15	2.430	0.194	—	15
April	2.397	0.232	0.249	11	2.491	0.179	—	11
June	2.212	0.644	0.319	10	2.507	0.167	—	10

Collector ropes:

Comparison among slopes, F = 1.091 (DF = 3,38) $P > 0.05$.

$b_{common} = 2.384$.

Comparison among intercepts, F = 12.944 (DF = 3,41) $P < 0.001$.

Rocky shore:

Comparison among slopes, F = 3.134 (DF = 3,38) $P < 0.05$

a and b are parameters in the equation $DW_{total} = aL^b$ (see Table 4, top). Rec. a represents recalculated intercept for common slope.

Although our results show a difference in growth rates between collector rope and rocky shore mussels, as was previously observed by Pérez Camacho et al. (1995), it should be pointed out that these differences become more apparent in those months that most favor growth (April through June), which is precisely the period in which the experiment by Pérez Camacho et al. (1995) took place, and just as was the case in their experiment, the most marked differences in our results are those for growth in wet weight and tissue weight. A higher growth efficiency for collector mussels when environmental conditions (temperature and overall quality of food) are more favorable, resulting in a more positive scope for growth, and the persistence of different metabolic patterns due to immersion-emersion periods that are indicative of anaerobic pathways for rocky shore mussels, could help us to understand such different growth responses. Genetic factors could also explain a significant proportion of the variances in production/growth of mussels (Widdows et al. 1984, Mallet et al. 1987), since it has been described that mussels exhibit high levels of genetic variability measured as enzyme polymorphisms both on a micro- and a macrogeographic scale (see Hawkins and Bayne 1992). Additionally, energy-saving mechanisms related to respiration metabolism have been described for those animals, which live in the intertidal locations. Metabolic depression and anaerobiosis are clearly implicated as key factors of energy conservation to withstand emersion conditions in order to compensate for reduced

feeding time with respect to sublittoral animals (de Zwaan and Mathieu 1992).

Both terms (physiological rates and metabolic patterns) are being tested for both types of seed mussel cultivated on suspended conditions in Arousa.

The use of the allometric function weight-length in growth studies is firmly established (Hickman 1979, Rodhouse et al. 1984, Sprung 1995, Sará et al. 1998, among others). A correspondence has occasionally been established between the variation in the allometric coefficient and local food conditions (Sará et al. 1998). In this study, we did not measure the original weight-length relationship of intertidal mussels before putting them on the raft. However, the fact that the experiment began a few hours after the seed mussels were gathered from their environments suggests that this relationship is similar to that which these mussels might show in their original habitat. An ANCOVA that was performed for this weight-length relationship in rocky shore mussels with regard to cultivation time period showed changes in the b parameter value (more evident between slopes of November and April), whereas collector rope mussels presented no differences in this value throughout the cultivation period. This probably means that environmental changes for rocky shore mussels, when they are put under immersed conditions on the raft, might be responsible for such a response of the allometric functions.

The initial differences in the CI of the two types of mussel seed can be attributed to their original habitats, which differ greatly with regard to the availability of food and their respective situations of emersion-immersion. The disappearance of these differences after 70 days may be related to the changes in physiological responses resulting from a new environmental situation (Bayne et al. 1984, 1987), although these differences may well persist for some time (Widdows et al. 1984, Iglesias et al. 1996).

Given the experimental design in this study, any effects on our results of a genetic nature that have occasionally been used to explain differences in growth (Peterson and Beal 1989, Rawson and Hilbish 1991) would be possible when genetic factors play a part in the choice of substrate (rocky shore or collector rope) by larvae in the Ría de Arousa or when different cohorts are involved. As has previously been mentioned by Pérez Camacho et al. (1995), an alternative hypothesis would be to consider a physiological adaptation response of each seed to its habitat of origin, which would imply that cultivation starts from different physiological states, which is described by Mallet et al. (1987) as ecological memory. This ecological memory would condition the physiological response of the seed to its new environmental situation, as shown by an increase in the CI for the rocky shore seed.

We can consider that the aim of slowing down the initial growth rates in order to minimize any possible advantages for the collector rope mussels has been achieved. This would explain why the differences observed in growth rates between the two types of seed are less marked than those recorded by Pérez Camacho et al. (1995) during the first stage of the cultivation period. Although the allometric coefficients for both types of seed need to be tested with regard to their original habitat for establishing more properly habitat-dependent changes, the CI differences maintained in both seed types supports the hypothesis that there is an underlying physiological basis for the difference in their respective growth rates. Moreover, given the experimental conditions under which the present study was performed and taking into account the CI changes, the physiological parameters of the two types of seed could be expected to converge.

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FEEDING BEHAVIOR OF SEED MUSSEL *MYTILUS GALLOPROVINCIALIS*: ENVIRONMENTAL PARAMETERS AND SEED ORIGIN

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ABSTRACT Mussel seed (*Mytilus galloprovincialis*) from two original habitats (rocky shore and collector ropes) was cultivated on a raft in the Ría de Arousa (northwest Spain), for a period of 226 days (November 1995 through June 1996), from seeding to thinning out, during which time the behavior of clearance rates (CR) and ingestion rates (IR) was studied. The study of these two physiological parameters of energy acquisition (CR and IR) demonstrates that the two types of seed showed significant differences in these parameters at the start of the experiment and after the first 8 days on the raft. After 15 days, large increases in these physiological rates were observed for both types of seed, with the increase for the rocky shore mussels doubling that of the collector rope specimens. These increases led to the disappearance of the significant differences in CR and IR between both seed origins, with this situation being maintained for the remainder of the experimental period. The variation in CR follows a seasonal pattern, with low values being recorded in winter and increasing in spring and summer. Minor seasonal variations of total seston concentration are counterbalanced by an inverse variation in organic content, and so organic IR followed a pattern similar to that of CR. This seasonal variation can be attributed to fluctuations in the factors food quality (Q_1) and temperature, in this order, as the use of multiple regression analysis has proved. Seed origin had a significant effect as a factor of interaction with food quality Q_1, probably because of differences between the original habitats of the seed (rocky shore and collector ropes) in the latter factor. Although in this study food quality has been expressed in terms of organic content (Q_1 = organic/total particulate matter), the content of phytoplankton as chlorophyll *a* may have had an important effect on the variation of both of these physiological rates. A significant exponential relationship has been established between the IR and the content in total particulate matter, which suggests regulation processes according to the amount of natural food available based on a decrease of CR.

KEY WORDS: *Mytilus galloprovincialis*, mussel seed, clearance rate, and ingestion rate, Ría de Arousa

INTRODUCTION

Clearance rates (CRs) and ingestion rates (IRs) determine the amount of food that enters the digestive system of bivalve molluscs. The variability observed in these physiological parameters has been interpreted in terms of the ability of these animals to adapt to the specific environmental and nutritional conditions of their habitat (Widdows et al. 1984; Navarro et al. 1991; Okumus and Stirling 1994; Iglesias et al. 1996). The relationship between IR and food concentration depends on CR, which in turn is affected by environmental factors. Hawkins and Bayne (1992) proposed the use of multifactorial analyses to ascertain the relevance and ecological complexity of the set of environmental variables, as well as their interaction with physiological parameters.

When attempting to determine the extent of the influence of habitat, transplant experiments are considered to be the ideal way of analyzing the effect of the variability attached to the environment in which the individuals lived previously, in connection with what Mallet et al. (1987) termed ecological memory. Previous comparative studies of mussel seed gathered from a rocky shore and from collector ropes and then cultivated on a raft established the existence of a significant effect of the seed origin on growth rate (Pérez Camacho et al. 1995; Babarro et al. 2000), with this effect being associated with physiological parameters.

The extent of time needed for CR and IR to acclimate to new environmental conditions has been reported in various studies (4.5 mo [Okumus and Stirling 1994] and more than 2 mo [Widdows et al. 1984], although Hawkins and Bayne [1992] have suggested a period of less than 2 mo). The aim of the present study was to determine the extent to which differences in the feeding regime and the regime of immersion-emersion in their original habitats (rocky shore and collector ropes) affects the behavior of CR and IR during the cultivation period in the raft (20–60-mm shell length). The study also deals with a set of factors, such as an endogenous factor (i.e., shell length) and the environmental and nutritional conditions in the cultivation area, and the effect they have on these physiological rates for raft-cultivated mussels.

MATERIALS AND METHODS

Harvesting and Maintenance of Mussels

In November 1995, seed of *Mytilus galloprovincialis* of approximately 20 mm in length was gathered from the rocky shore and from collector ropes on a raft, both in the mid to outer area of the Ría de Arousa (Galicia, northwest Spain). Both types of seed, from the same year class, came from a spawning period in the previous spring/summer. Experimental cultivation, which was carried out under production conditions on the raft (500 m^2), began in winter—the season of minimal growth rate—with the aim of minimizing any possible advantages for the collector rope seed as a result of its better adaptation to raft cultivation conditions. The experiment continued until July 1996 (226 days) and covered the first stage of mussel cultivation from seeding to thinning out (50–60 mm). Sixteen cultivation ropes (12 m) were used, 8 for each type of seed, disposed alternately and with a density of 19 kg of seed per rope (1.6 kg/m of rope or 2,600 individuals/m of rope). Specimens were sampled each time from adjacent ropes from the stretch of 2–5 m.

The initial length of the seed was 22.55 ± 1.55 mm for collector rope seed and 19.02 ± 1.93 mm for the rocky shore seed. Mean total dry weight was 0.36 ± 0.06 and 0.27 ± 0.06 g/individual for each type of seed, respectively. These differences in length and dry

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weight between the mussels from the two different original habitats were found to be not significant at the beginning of the experiment (analysis of variance [ANOVA]; $P > 0.05$ in both cases; $n = 96$).

Experimental Design

Seawater was pumped from the depth where seed was sampled (2–5 m) into an open circuit consisting of three rectangular cages (45 × 40 × 14 cm = length × width × height and 19 L of capacity), each provided with 16 compartments set in parallel. Seed specimens from the two origins were placed in the side cages while the middle cage, containing no specimens, acted as the control. The water flowed independently into each cage from an inlet pipe, which went all the way around the top of the cage. The water outlet for each cage consisted of a single pipe leading off from the top of the cage. The flow in each cage was maintained at a steady rate of approximately 3 L/min, so that the concentration of particles at the outlet would never fall below 50% of that at the inlet. The number of specimens used in each replica for physiological measurements varied according to their size (i.e., with the length of cultivation period). At the outset, six specimens of the 20-mm shell length class were placed in each compartment, and this number was also used for 30-mm shell length. From the 40-mm shell length onward, the number of animals used progressively decreased and at the end of the experiment there was only one specimen of the 60-mm length class in each compartment. Physiological measurements were taken weekly from November to January, fortnightly from February to May, and monthly in June and July.

Measurements

Natural seston was characterized as total particulate matter (TPM, mg/L), particulate organic matter (POM, mg/L), particulate inorganic matter (PIM, mg/L), particulate volume (Vol, mm^3/L), and chlorophyll *a* (chl-*a*, μg/L). The values for chl-*a*, as well as for the temperature (°C) and salinity (‰) of the water column were provided by the Centro de Control de Calidade do Medio Mariño da Consellería de Pesca, Marisqueo e Acuicultura (Xunta de Galicia). chl-*a* was calculated from the fluorescence data. Seston quality was expressed as Q_1 (POM/TPM), Q_2 (POM/Vol), and the chl-*a*/TPM index. The same methodology as that used for gravimetric analysis of seston was applied to characterize the feces produced by the mussels in the experimental system: seawater samples and aliquots of known volumes from each fecal sample were filtered onto pre-ashed (450 °C for 4 h) and weighed GFC filters and rinsed with isotonic ammonium formate (0.5 M). Total dry matter was established as the weight increment determined after drying the filters to constant weight at 110 °C for 12 h. Organic matter corresponded to the weight loss after ignition at 450 °C for 4 h in a muffle furnace. Vol/L of seawater was determined by counting in the range of 2–56 μm using a Coulter Counter Multisizer II fitted with a 100-μm aperture tube. The variation in these environmental and/or nutritional parameters over the cultivation period is shown in Table 1.

The egestion rates of inorganic matter (mg/h) were determined for each group of mussels and assumed to represent inorganic IR (i.e., no absorption of ash in the digestive tract was considered). CRs were then estimated indirectly, with PIM concentration (mg/L of seawater) as the reference for available inorganic matter: then $CR\ l\ h^{-1}$ = mg $PIM_{Feces}\ h^{-1}$/mg $PIM_{Food}\ L^{-1}$ (Iglesias et al. 1996; 1998), where PIM_{Feces} is the amount of inorganic content voided with the feces in a given unit of time (h) and PIM_{Food} is the inorganic content of the food in a given unit of volume (L). A lag time of 2 h was allowed between the sampling of seawater and the gathering of feces, to account for the estimated time for intestinal transit for mussels from the Ría de Arousa (Navarro et al. 1991). Before the start of the experiments, mussels were kept in the cages for 1 h with flowing seawater at the natural particle concentration to allow for valve opening and acclimation to cage conditions. The feces obtained on the bottom of cages after this time were refused.

The organic ingestion rate (OIR mg org/h) was calculated as a product of CR and the organic food concentration (mg POM/L).

For purposes of comparison with this indirect CR estimation, CR was also calculated by the direct estimation (flow method) using the Hildreth and Crisp (1976) equation: $CR = f([C_i - C_o]/C_m)$, where f is the flow rate, C_i and C_o are food concentrations at the inflow and outflow of the experimental cage, and C_i represents the particle concentration surrounding the mussel. The experimental design used in this study enabled us to consider C_m concentration as being close to C_i, so C_i was subsequently used as the reference concentration for calculation purposes. C_i and C_o were determined by recording the concentration of particles 2–56 μm in water samples with a Coulter Counter Multisizer.

The degree of correlation obtained between the two calculations for CR was highly significant for both groups of mussels together, similar to those observed by Urrutia et al. (1996) and Iglesias et al. (1998) (Y [biodeposition] = 1.118 ± 0.074 × flow + 0.043; $r^2 = 0.86$; $P < 0.001$; $n = 300$). Once significant relationships were established for both methodologies, CR here (text, tables, and figures) refers to indirect measurements by biodeposition method.

Size Standardization

To preclude variability in physiological rates caused by size differences, these rates were corrected to a standard-sized individual. To this end, once physiological measurements were completed, shell length of each individual was recorded to the nearest 0.1 mm with Vernier calipers and the soft tissues excised from the shell, dried at 110 °C for 12 h, and weighed. The most commonly used reference for size is soft body mass; however, the weight standardization of CR may be somewhat arbitrary, because this rate is considered to be dependent on filtration (gill) area, which is closely related to shell length (Hughes 1969; Jones et al. 1992). As discussed before by Iglesias et al. (1996) and Labarta et al. (1997), in this study we used shell length (L) to standardize CR following the equation: $Y_s = Y_e \times (L_s/L_e)^b$ where Y_s and Y_e are the standardized and the nonstandardized CRs, respectively; L_s is the standard length of the animal according to shell increment during the experiment (20–60 mm); L_e is the observed length of the animal; and b is the power that scales CR with shell length (b = 1.85, Pérez Camacho and González 1984). Furthermore, with the aim of establishing the fluctuation of clearance and IRs over the cultivation period, 40-mm shell length was chosen as an average size for the experiment.

Data Analysis

Comparison of means for CR and OIR was carried out by means of standard ANOVA after data transformation when necessary. Kruskall-Wallis and Friedman nonparametric tests were used when homogeneity was lacking (Bartlett's test). Multiple analysis (stepwise regression) was used to determine the effect of

TABLE 1.

Characteristics of the natural seston.

Date	TPM (mg/L)	POM (mg/L)	PIM (mg/L)	Vol (mm^3/L)	Temperature (°C)	Salinity (‰)	Chl-*a* µg/L	Q_1 (POM/TPM)	Q_2 (POM/Vol)	Chl-*a*/TPM
11/27/95	0.677 ± 0.183	0.373 ± 0.106	0.304	0.570 ± 0.287	15.649 ± 0.703	32.838 ± 1.960	0.754 ± 0.184	0.551	0.654	1.114
12/5/95	0.488 ± 0.151	0.290 ± 0.044	0.198	0.389 ± 0.207	15.398 ± 1.078	31.307 ± 4.734	0.362 ± 0.267	0.594	0.746	0.742
12/13/95	0.832 ± 0.114	0.314 ± 0.027	0.518	0.524 ± 0.087	15.557 ± 0.450	35.157 ± 0.499	0.476 ± 0.151	0.377	0.599	0.572
12/20/95	0.736 ± 0.212	0.373 ± 0.076	0.363	0.455 ± 0.084	13.683 ± 0.408	34.761 ± 0.368	0.609 ± 0.146	0.507	0.820	0.827
1/3/96	2.561 ± 0.676	1.003 ± 0.268	1.558	1.290 ± 0.086	14.264 ± 0.261	30.066 ± 2.950	0.365 ± 0.358	0.392	0.778	0.143
1/17/96	0.792 ± 0.056	0.464 ± 0.025	0.328	0.515 ± 0.078	13.925 ± 0.646	28.010 ± 4.767	0.286 ± 0.282	0.586	0.901	0.361
1/31/96	0.549 ± 0.073	0.278 ± 0.032	0.271	0.292 ± 0.048	13.225 ± 0.287	30.130 ± 1.401	0.644 ± 0.268	0.506	0.952	1.173
2/15/96	1.342 ± 0.198	0.387 ± 0.056	0.955	0.712 ± 0.063	13.861 ± 0.650	31.058 ± 2.172	1.434 ± 0.355	0.288	0.543	1.069
2/28/96	1.310 ± 0.111	0.566 ± 0.076	0.744	1.066 ± 0.143	12.914 ± 0.422	34.136 ± 1.363	1.162 ± 0.180	0.432	0.531	0.887
3/13/96	0.881 ± 0.224	0.459 ± 0.102	0.422	0.899 ± 0.288	13.152 ± 0.081	34.906 ± 0.352	1.187 ± 0.371	0.521	0.511	1.347
3/27/96	0.962 ± 0.175	0.522 ± 0.085	0.440	1.262 ± 0.264	13.832 ± 0.237	33.059 ± 0.415	1.708 ± 0.277	0.543	0.414	1.775
4/10/96	1.381 ± 0.119	0.643 ± 0.036	0.738	1.663 ± 0.147	14.412 ± 0.476	32.532 ± 2.688	1.957 ± 1.601	0.466	0.387	1.417
4/24/96	0.742 ± 0.086	0.394 ± 0.025	0.348	0.655 ± 0.150	15.018 ± 0.570	33.685 ± 1.717	1.595 ± 0.352	0.531	0.601	2.150
6/5/96	0.623 ± 0.090	0.289 ± 0.038	0.334	0.765 ± 0.098	15.043 ± 1.208	34.970 ± 0.960	1.115 ± 0.589	0.464	0.378	1.790
7/3/96	0.179 ± 0.130	0.432 ± 0.083	0.287	0.644 ± 0.161	14.335 ± 0.686	35.586 ± 0.160	1.030 ± 0.299	0.601	0.671	1.433

TPM, total particulate matter; POM, particulate organic matter; PIM, particulate inorganic matter; Vol, total particulate volume; Chl-*a*, chlorophyll-*a*; Q_1, relative organic content (by weight); Q_2, relative organic content (by volume); Chl-*a*/TPM, relative chlorophyll-*a* content (by total particulate matter).

various factors, both endogenous (shell length) and environmental (TPM, POM, Vol, Q_1, Q_2, T, and chl-*a*), and their interactions on the variation in CR. The factor seed origin was added to this analysis with values of 0 and 1 for collector rope and rocky shore mussels, respectively. All of these analyses were performed according to the methods described by Snedecor and Cochran (1980) and Zar (1984).

RESULTS

The values of CR and OIR during the experiment are shown in Table 2. CR ranges between 0.20 and 0.26 L/h in the initial stages of cultivation (20-mm mussel), rising up to 4.37–4.51 L/h for a 60-mm mussel.

CR values for collector rope mussels were significantly higher than those of rocky shore mussels in days 0 and 8 of the experimental period ($P < 0.01$ in both cases; ANOVA; Table 2). However, CR for the rocky shore mussels had increased by 35% by the end of the 2nd week, compared with only 16% for the collector rope specimens. From this point on, no further significant difference in this physiological rate was recorded between the two types of mussels ($P > 0.05$; Table 2). In the case of OIR, both groups of mussels followed the same pattern as that described for CR (Table 2).

VARIATION OF CR AND OIR IN CULTIVATION TIME

Figure 1 shows the variation of standardized CR and OIR for the 40-mm length class in cultivation time. CR showed a clear seasonal pattern with low values during the winter months, increasing in spring and summer. High values recorded in January constituted the sole exception. These trends were also recorded for OIR (Fig. 1), amplified in this case by the coincidence of high CR and high POM.

CR versus Shell Length and Environmental Parameters

The multiple regression analysis carried out on the variation observed in CR during the experiment showed a significant and positive relation to size (L mm), food quality (Q_1), and temperature (see F-ratio, Table 3). The regression model accounted for 76.7% of the variance for CR, which in turn is mainly accounted for by size (L mm 67.6%), with a coefficient of 1.762. It is important to point out the significant negative effect of the interactions of food quality (Q_1) with both origin ($Q_1 \times$ origin) and temperature ($Q_1 \times T$) (Table 3).

Organic IR versus Natural Seston (TPM mg/L)

A significant relationship was established between the IR (OIR) and the variation in seston (TPM, mg/L), that could not be established in the case of the CR. This response of ingestion to seston concentration is shown in Figure 2 and fits exponential functions according to the Ivlev curves $IR = a\,(1 - e^{b \cdot TPM})$:

Collector ropes

$$OIR = 1.29 \pm 0.39\,[1 - e^{-0.75 \pm 0.36 \cdot TPM}]$$
$$n = 14;\ r^2 = 0.517;\ P < 0.01$$

Rocky shore

$$OIR = 1.18 \pm 0.36\,[1 - e^{-0.87 \pm 0.46 \cdot TPM}]$$
$$n = 14;\ r^2 = 0.481;\ P < 0.01$$

The covariance analysis performed for the linear transformations of these exponential curves showed no significant differences

TABLE 2.

Values of physiological parameters (mean ± SD, n = 32) of two sources of seed muscles standardized to shell length (L) according to growth of *M. galloprovincialis* during the experiment.

Date	Cultivation Days	Source of Seed Mussel	L (mm)	CR (L/h)	OIR (mg POM/h)
11/27/95	0	Collector ropes	20	0.43 ± 0.12*	0.16 ± 0.04*
		Rocky shore		0.34 ± 0.09	0.13 ± 0.03
12/5/95	8	Collector ropes	20	0.26 ± 0.09*	0.08 ± 0.03*
		Rocky shore		0.20 ± 0.09	0.06 ± 0.02
12/13/95	15	Collector ropes	20	0.50 ± 0.10	0.16 ± 0.03
		Rocky shore		0.46 ± 0.15	0.14 ± 0.05
12/20/95	22	Collector ropes	20	0.37 ± 0.06	0.14 ± 0.02
		Rocky shore		0.35 ± 0.08	0.13 ± 0.03
1/3/96	36	Collector ropes	20	0.40 ± 0.09	0.40 ± 0.09
		Rocky shore		0.36 ± 0.10	0.36 ± 0.10
1/17/96	50	Collector ropes	20	0.57 ± 0.14	0.26 ± 0.06
		Rocky shore		0.60 ± 0.15	0.28 ± 0.07
1/31/96	64	Collector ropes	30	0.72 ± 0.23	0.20 ± 0.06
		Rocky shore		0.71 ± 0.25	0.20 ± 0.07
2/15/96	80	Collector ropes	30	0.49 ± 0.09	0.19 ± 0.04
		Rocky shore		0.52 ± 0.15	0.20 ± 0.06
2/28/96	95	Collector ropes	30	0.69 ± 0.13	0.39 ± 0.07
		Rocky shore		0.69 ± 0.14	0.39 ± 0.08
3/13/96	110	Collector ropes	40	1.23 ± 0.27	0.56 ± 0.13
		Rocky shore		1.25 ± 0.38	0.57 ± 0.18
3/27/96	125	Collector ropes	40	1.13 ± 0.27†	0.59 ± 0.14†
		Rocky shore		1.27 ± 0.29	0.66 ± 0.15
4/10/96	140	Collector ropes	40	1.16 ± 0.47	0.74 ± 0.30
		Rocky shore		1.17 ± 0.23	0.75 ± 0.15
4/24/96	155	Collector ropes	50	2.47 ± 0.74	0.97 ± 0.29
		Rocky shore		2.06 ± 0.64	0.81 ± 0.25
6/5/96	197	Collector ropes	60	4.51 ± 1.21	1.30 ± 0.35
		Rocky shore		4.37 ± 1.16	1.26 ± 0.33
7/3/96	226	Collector ropes	60	3.92 ± 0.89	1.69 ± 0.38
		Rocky shore		4.09 ± 0.83	1.77 ± 0.36

CR, clearance rate length-specific (L/h) by biodeposition method; OIR, organic ingestion rate length-specific (mg POM/h).
* $P < 0.01$, †$P < 0.05$, ANOVA and Kruskall-Wallis nonparametric test in case of heterogeneity of variances).

between both groups of mussels for OIR ($t = 0.037$, $df = 24$, $P > 0.05$, and $t = 0.358$, $df = 25$, $P > 0.05$ for analysis of slopes and intercepts, respectively). Therefore, one exponential curve for both groups of mussels together is shown in Figure 2:

$$OIR = 1.23 \pm 0.26\,[1 - e^{-0.78 \pm 0.26 \cdot TPM}]$$
$$n = 28;\ r^2 = 0.500;\ P < 0.01$$

DISCUSSION

The variation in CR and OIR during the experimental period, for mussels standardized to 60 mm to compare with the literature values, covers a wide range (1.46–4.51 and 1.13–4.37 L/h for CR, 0.43–2.23 and 0.33–2.04 mg POM/h for OIR, values for collector rope and rocky shore mussels, respectively). These data coincide with those obtained by Navarro et al. (1991) and Iglesias et al. (1996) for *M. galloprovincialis* in the Ría de Arousa. In the case of CR, however, these values are higher than those recorded for mussels elsewhere reported by Okumus and Stirling (1994) in their wide-ranging review. Despite the above-mentioned differences in CR due to low seston loads, characteristics from Galician Rias in particular, the values for organic IRs reported in the present study are similar to those obtained by Widdows et al. (1979), also under environmental conditions, and by Bayne et al. (1989) in the laboratory, with a higher range of values for seston and organic content (0.79–7.43 mg TPM/L, 0.43–1.79 mg POM/L, and 0.18–0.71 for Q_1).

The few studies that include CR data recorded over a period of seasons show that CR follows a clear seasonal pattern, with maximum values occurring in the spring and summer months and minimum values in winter. This cycle can be observed both under laboratory conditions, with a constant food supply available (Worrall et al. 1983), and under natural conditions (Newell and Bayne 1980). Larretxea (1995), taking into account a previous study of Hawkins et al. (1985), suggests that the seasonal sequence of CR is persistent to a large extent, although the effect of temperature could be an important determinant of this seasonal response.

In this study, rates of energy acquisition exhibit a seasonal pattern, with lower values occurring during the winter months and slightly higher during spring and summer. The range results greater in terms of OIR because of the simultaneous decrease in CR and in organic content of the seston. An exception to this overall behavior are those values found to deviate largely from the mean of the season during which they were obtained, namely unexpectedly high values for CR, and especially OIR, in the samples taken in January. These may be accounted for by the high

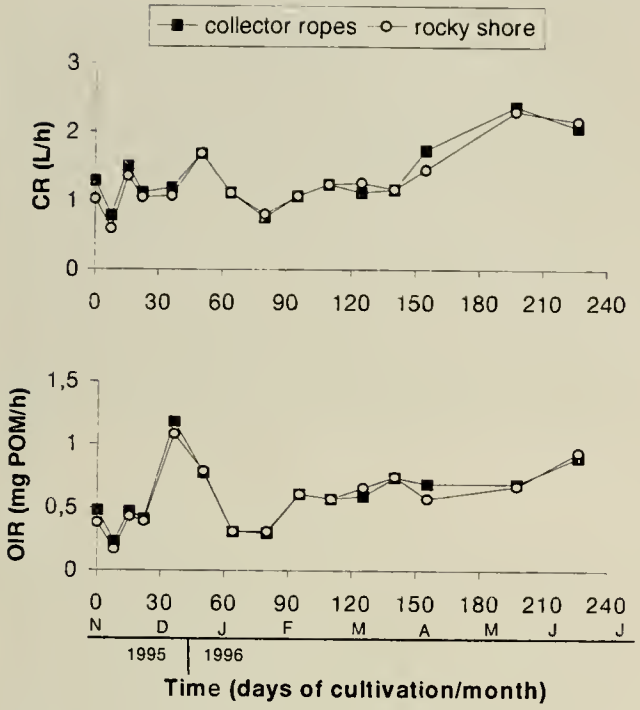

Figure 1. Seasonal changes in CR and OIR standardized to 40 mm of shell length for both sources of seed mussel *M. galloprovincialis*.

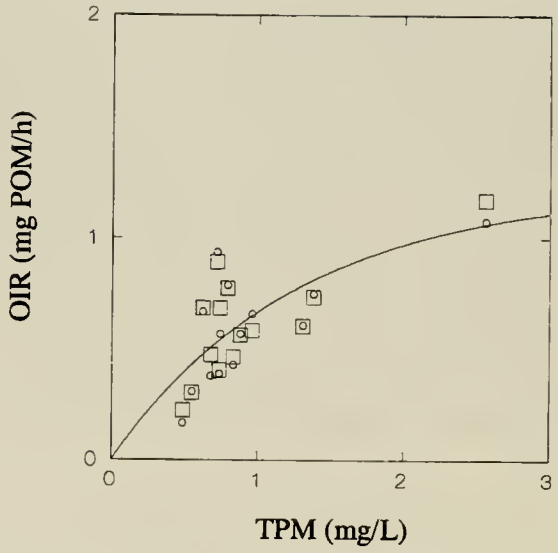

Figure 2. OIR versus TPM relationship for both sources of seed mussel *M. galloprovincialis*. Both groups of mussels (collector ropes, squares; rocky shore, circles) were fitted by nonlinear regression according to Ivlev curve: $Y = a(1 - e^{b \cdot X})$ (see text for details of fitting equation).

POM values (1.003 mg/L) and high Q_1 value (0.586), which can be considered as a result of a process of resuspension of material from the bottom in the Ria de Arousa (Babarro et al. 2000). Similar feeding behavior has been observed by Kiørboe et al. (1981) and Larretxea (1995) concerning CR increments associated with an increase in detritus and sediment resuspended.

With regard to seston composition, the results of this study show a 33% increase in total organic content in spring/summer when compared with winter, which can be related to an increase in mean CR of 30% for collector rope mussels and 40% in the case of rocky shore specimens.

However, other factors that seem to exert indirect influence on the energy gain should be taken into account. This is the case for chl-*a* values for the period February through July that doubles that of the period November through February (Babarro et al. 2000). During the winter months, the proportion of phytoplankton (chl-*a*) in the organic content of the diet is 4–23%, rising to 21–41% during spring and summer, with peaks of 37.4% and 40.9% in April and February, respectively, which bears a close relationship to the seasonal variation in CR and OIR (carbon content = chl-*a* × 54, Widdows et al. 1979; organic matter = carbon × 1.87, Fernández Ríos 1992). This incidence of phytoplankton (chl-*a*) can also be observed in the fact that when the value for POM is not associated with chl-*a* (Babarro et al. 2000), no effect on CR is observed. However, the effect of this factor (chl-*a*) has not been tested in the multiple model because of the use of Q_1 as a factor of food quality and in order to avoid overlapping of information.

TABLE 3.

Stepwise multiple regressions of clearance rate of mussels with log shell length (L), quality of seston (Q_1 = POM/TPM), temperature (T, °C), and interactions terms.

Parameter	Coefficient	SE	F-Ratio	*P*	r^2
Constant	−20.329				
Log L	1.762	0.042	1764.516	<0.001	0.676
$Q_1 \times T$	−1.297	0.085	230.838	<0.001	0.683
$Q_1 \times$ origin	−0.070	0.023	9.491	<0.01	0.686
T	1.778	0.245	52.512	<0.001	0.688
Q_1	18.948	1.228	238.067	<0.001	0.761
T^2	−0.039	0.009	20.776	<0.001	0.767

$r^2 = 0.767$; $n = 812$; $F_{6,805} = 440.729$; $P < 0.001$

Origin factor has been estimated with values 0 and 1 for collector ropes and rocky shore mussels, respectively.

The multiple regression model for CR shows the importance of shell length, food quality (Q_1), and the temperature either as an independent variable or as a term in interaction ($Q_1 \times T$). Very likely the presence of a term $Q_1 \times$ origin could be related to differences in seston quality between both original locations. Previous studies carried out with both groups of mussels showed higher Q_1 values for subtidal location than that for rocky shore (unpublished data). Shallow water and stronger tidal waves in the rocky shore spot seem to affect the relation organic:inorganic fraction, with resuspension processes of the sediment playing an important role. Mussels seem to adjust their feeding rates in a relatively short time under environmental changes (first 8 days under culture conditions), and probably when animals are "adapted," fluctuations of Q_1 after this initial period of time do not cause different CR responses between the two populations.

The effects of the food ration or particle concentration on filtration rates in bivalve molluscs have been widely studied over the years. A reduction in CR when seston concentration increase has been reported in several experiments (Foster-Smith 1975; Widdows et al. 1979; Riisgård and Randløv 1981). As was previously established by Winter (1978), it seems that the ability of bivalves to adjust CR in response to an increase in particle concentration allows the regulation of IR.

In fact, the relationship between IR and seston concentration has been appropriately described by an exponential asymptotic

function (Ivlev curve) in this study. This behavior suggests to us a mechanism of regulation of ingestion based, in this case, on adjusting CR and taking into account that this saturating increase in OIR cannot be assigned to the negative organic content versus seston availability relationship, which was not observed in our study as significant. Although higher CRs are related with lower seston availability values, significant effects of either TPM or POM and chl-*a* on CR were not observed, possibly because of the reduced range of variation in seston concentration.

As already been mentioned, the results of this study establish that temperature has a significant effect on CR. The thermodependence of CR coincides with the observations made by Widdows (1976), namely that mussels living in a stable thermal environment (which is the case with a range of temperature variation of 2.73 °C) have not developed compensation mechanisms, being thermodependent.

Although the effects associated with origin have been considered by many authors to be indicative of genetic differences between mussels from different original habitats, Mallet et al. (1987) offered an alternative explanation. In their study, the authors suggested that these effects would reflect the differential influences undergone by mussels during their pre-experimental stage, so conforming to an "ecological memory" of the individuals with respect to the conditions experienced in the primary habitat (food availability and quality, tidal regime, air exposure, etc.). Okumus and Stirling (1994), Navarro et al. (1996), Iglesias et al. (1996), and Labarta et al. (1997) all recorded differences in CR for mussels from different original habitats, which they attribute to this ecological memory.

The present study shows significant differences for CR and OIR between different original habitats of the mussels (collector rope and rocky shore) at the outset of the experiment that persisted after 8 days' cultivation on the raft. These significant differences concerning the two physiological rates between both types of mussel disappeared 15 days after raft cultivation commenced. The initial differences in CR and OIR may be the consequence of a response by the rocky shore mussels to the new conditions found on raft cultivation (i.e., a lower concentration and higher-quality Q_1 of seston in continuous immersion; previous data unpublished) over a short period of time. This hypothesis is supported by results of the multiple regression analysis, which indicates that both Q_1 and origin, the latter being expressed as a term of interaction with Q_1, account for a part of the variance experienced by CR according to the model.

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SALINITY TOLERANCE OF BROWN MUSSEL *PERNA PERNA* (L.) FROM THE GULF OF MEXICO: AN EXTENSION OF LIFE TABLE ANALYSIS TO ESTIMATE MEDIAN SURVIVAL TIME IN THE PRESENCE OF REGRESSOR VARIABLES

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ABSTRACT The nonindigenous brown mussel *Perna perna* was first recorded in the Gulf of Mexico at Port Aransas, Texas in 1990. The association between survival time and chronic exposure to hypo- and hypersaline conditions was examined to estimate the potential range of habitats that *P. perna* could invade in coastal North American Gulf of Mexico habitats. A novel application of the discrete logistic failure time model (DLFTM) was used to estimate covariate-adjusted median survival times from the interval-level survival data collected. This method allowed factorial-type comparisons of the covariate-adjusted medians across treatments. This analysis indicated that salinities ranging from 15–50 ppt are nonlethal to *P. perna*, under which at least 80% of individuals survived 30 days (720 h). Chronic exposure to salinities outside 15–50 ppt were lethal to *P. perna*. Lethality was size-dependent, with both smaller and larger individuals having reduced survival times. For an average-sized individual (shell length = 40 mm), median survival times were 191, 268, 335, 119, and 116 h at 0, 5, 10, 55, and 60 ppt, respectively. The 15–50 ppt incipient salinity limits of Texas *P. perna* suggest that this species could potentially colonize the majority of marine and estuarine coastal habitats in the Gulf of Mexico.

KEY WORDS: interval-level survival data, mytilacea, nonindigenous species, *Perna perna*, salinity tolerance

INTRODUCTION

Since its initial discovery in Port Aransas, Texas, in 1990, the marine brown mussel, *Perna perna* (L.), has colonized hard shores at intermittent locations throughout the southwestern Gulf of Mexico in Texas and Mexico (Hicks and Tunnell 1993, Hicks and Tunnell 1995). The majority of the Gulf of Mexico's margins are sandy beaches, which has historically impeded development of natural hard-shore communities. Thus, it was not until the construction of jetties, breakwaters, and other coastal structures during the last century that habitat existed for true intertidal bed-forming mytilid genera such as *Perna*. These man-made structures, which are continuously being constructed to control coastal erosion, present a relatively open niche for such invasive mytilid species as *P. perna*. Generally, considered an open-water species (Berry 1978), Texas *P. perna* populations have been reported from littoral and shallow sublittoral habitats of widely varying physiochemical parameters including the low salinity (20–25 ppt) Lavaca-Tres Palacios estuary (Davenport 1995) and the hypersaline (35–40 ppt and occasionally higher) Laguna Madre (McGrath et al. 1998).

The endemic range of *P. perna* (synonymous with *Perna picta* (Born) and *Perna indica* Kuriakose and Nair, [Siddall 1980, Vakily 1989]) includes India, Sri Lanka, Madagascar, the east coast of Africa from central Mozambique to False Bay, South Africa, and the African west coast from Luderiz Bay, Nambia, north into the Mediterranean from Gibraltar to the Gulf of Tunis as well as on the Atlantic coasts of Brazil, Uruguay, Venezuela, and in the West Indies (Berry 1978).

Salinity influences many physiological functions, making it an important limiting factor in the distributions of estuarine and marine bivalves (Bayne et al. 1976, Widdows 1985, Dame 1996). As with most marine mytilaceans, *P. perna* is incapable of extracellular osmotic control; thus, its extracellular fluids are nearly isosmotic to ambient seawater over its tolerated salinity range (Salomão and Lunetta 1989). The typical short-term response of osmoconforming bivalves to salinity reductions is to close the shell valves temporarily isolating tissues and body fluids from potentially lethal hyposaline waters, while allowing time for intracellular volume regulation by adjusting the concentrations of intracellular amino acids and other small organic molecules (Hawkins and Bayne 1992).

We examined the effects of chronic exposure to hypo- and hypersaline media on survival times in *P. perna*. Salinity tolerance data have proved effective in predicting local distributions in marine bivalves (Castagna and Chanley 1973). Thus, the incipient salinity limits determined in this study were used to predict the potential for *P. perna* to colonize coastal Gulf of Mexico marine and estuarine habitats in North America.

We also developed a specialized methodology for analyzing our survivorship data that allows estimating and comparing covariate-adjusted median survival times for grouped lifetime data. Current methods for analyzing data of this type compare treatment survival distributions based upon odds-ratios. Our method of using median survival times, as opposed to odds, provides biologically more meaningful interpretations of survival data.

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MATERIALS AND METHODS

Experimental Protocol

Specimens of *Perna perna* were collected from intertidal rocks on the north jetty of Mansfield Pass (26 °34 ′N) on the Texas coast and were transported overnight in cooled insulated containers to Arlington, Texas. Upon arrival, mussels were maintained in a 284-L aerated holding tank containing artificial seawater (35 ppt) at a constant temperature of 20 °C on a 12:12 hour light–dark cycle without feeding before experimentation. Experiments were initiated within 30 days of collection.

Individual mussels were excised from mussel clumps by cutting byssal attachment threads with scissors before salinity tolerance testing. For each salinity tested, four subsamples of 10 mussels each, visually selected to be of similar size range, were held for 2 weeks in a constant temperature laboratory at 20 °C (±1 °C) in 3.5-L plastic aquaria containing 3 L of continuously-aerated, 35 ppt artificial sea water (Fritz Supersalt). Tank medium was replaced daily. During the 2 week acclimation period, mussels byssally reattached to tank walls or other individuals. The size range of the subsamples utilized in each salinity test reflected the size range in the original sample (shell lengths ~ 15–70 mm).

After acclimation to experimental conditions, subsamples were randomly assigned to test salinities of 0, 5, 10, 15, 20, 30, 40, 50, 55, and 60 ppt (created with Fritz Supersalt and City of Arlington, Texas, dechlorinated tap water), chosen to include the range of salinities occurring in Texas coastal aquatic habitats. Testing was initiated by replacing the 35 ppt seawater medium in each tank with 3 L of test salinity medium. During testing, media were held at 20 °C (±1 °C), continuously aerated and changed daily. Byssal attachment, valve opening or closure, and viability of all individuals was examined at 24-h intervals. Viability was determined by touching the exposed mantle edges of emersed gaping mussels with the tip of a fine-haired brush. Individuals not closing their valves in response to this stimulus were considered dead, and were removed from the aquaria. The time of the observation was recorded, and the shell lengths (SL) of dead individuals were measured as the linear distance from the anterior to posterior margins of the shell to the nearest 0.1 mm using digital calipers. All nongaping individuals were considered alive. Exposure to salinity treatments and viability testing was continued until either 100% sample mortality was achieved or individuals survived for 30 days (720 h).

Statistical Methods

The salinity resistance of specimens of *P. perna* was examined using a survival analysis strategy designed to determine the effects of seawater concentration on survival duration; whereas, controlling for individual-specific covariates (e.g., size). Our viability monitoring at 24-h intervals prevented knowledge of an individual's exact time of death. Thus, survival time was known only to fall within the interval $I_j = [a_{j-1}, a_j]$, while a_j was the current observation time, and a_{j-1} was the last observation time. Available parametric (e.g., Weibull) and nonparametric (e.g., Kaplan-Meier, Cox regression) survival estimation methods assume that the time of death is known exactly. However, in the vast majority of such studies, as in this one, time of death is not recorded exactly, but is known only to have occurred within a particular interval (Hosmer and Lemeshow 1989). Applying continuous-data methodology to such interval-level data can result in serious bias, especially if the interval length is large relative to the average lifetimes one is observing.

When survival data are recorded at the interval level, the life table (i.e., actuarial method) is often used to estimate survival probabilities. Life tables are essentially frequency tables modified to deal with censored observations (i.e., those data for individuals that survive treatments) (Lawless 1982). The main outcome of life table analysis is estimation of the survival function $S(a_j)$, which is the probability of surviving to time a_j, for all observation times $a_1, \ldots, a_k$. However, standard life table analysis cannot incorporate continuous regressors, such as size, which are likely to influence individual survival times. When such regressor variables are present, a discrete logistic failure time model (DLFTM), which generalizes the life table method, can be used to estimate survival probabilities and allow their adjustment for regressor effects (Cox 1972, Thompson 1977). The ability to include regressor variables in the DLFTM greatly broadens the scope of life table analysis by revealing both treatment and individual-specific regressor influences, allowing more biologically appropriate interpretations of survival data (Lawless 1982, Hosmer and Lemeshow 1989).

Although survival probabilities, when graphed into the usual form of survival functions, provide a summary of the survival experience of a population, these functions are cumbersome when there are many such populations to be compared (e.g., levels of treatments, different values of important regressors). In such cases, it is useful to have a one-number summary (e.g., the median survival time) of each survival function to compare across many populations. The DLFTM can, as illustrated here, be used to provide such median estimates for interval level data under some reasonable assumptions discussed below.

We implemented the DLFTM for our data analysis in a computer program written in SAS's interactive matrix language (IML, SAS, Cary, NC) available from the authors. The routine was checked for programming errors using simulations of data from lifetime distributions with known parameters.

DLFTM and Its Estimation

For our analysis, the 720-h observation period was divided into 24-h time intervals, $I_j = [a_{j-1}, a_j)$, $j = 1, \ldots, k = 30$, where $a_j = 24 \cdot j$ are times of observation, $a_0 = 0$ and $a_{k+1} = \infty$. The data for the ith individual, $1 \le i \le n$, consists of the vector $\mathbf{G}_i = (G_{i1}, \ldots, G_{i,k+1})$, where $G_{ij} = 1$ if individual i dies in interval I_j and $G_{ij} = 0$ otherwise, and a vector $\mathbf{x}_i$ of covariates describing treatments and individual-specific characteristics.

Let T_i denote the actual, but unobserved, lifetime of individuals. Let $S(t \mid \mathbf{x}) = \Pr(T_i > t \mid \mathbf{x})$ denote the survival function of individuals with regressor $\mathbf{x}$, which contains indicator variables describing salinity level and shell length (SL). The goal of the analysis was to estimate the survival probabilities $\underline{P}_j(\mathbf{x}) = S(a_j \mid \mathbf{x})$ at each of the observation times $a_1, \ldots, a_k$. By a standard argument using conditional probabilities (see Lawless 1982, p. 55),

$$\underline{P}_j(\mathbf{x}) = p_1(\mathbf{x}) \cdots p_j(\mathbf{x}), \quad 1 \le j \le k, \tag{1}$$

where $p_j(\mathbf{x}) = \Pr\{T_i \ge a_j \mid T_i \ge a_{j-1}, \mathbf{x}\}$ is the probability of surviving the time interval I_j, given alive at its outset. The $p_j(\mathbf{x})$s are called "interval-specific" survival probabilities. It is clear from (1) that to estimate $\underline{P}_j(\mathbf{x})$ it is enough to estimate the $p_j(\mathbf{x})$s.

The method of maximum likelihood estimation was applied to estimate the $p_j(\mathbf{x})$s, because it is known to produce estimates statistically optimal for large sample sizes n. In this regard, again by

a standard argument (see Lawless 1982, p.372), the likelihood function for the above data on n independent individuals, assuming censoring only at a_k, is

$$L[p_1(\bullet), \ldots, p_k(\bullet)] = \prod_{j=1}^{k} \left\{ \prod_{i \in D_j} [1 - p_j(\mathbf{x}_i)] \cdot \prod_{i \in R_j - D_j} p_j(\mathbf{x}_i) \right\} \quad (2)$$

where: R_j is the set of individuals i who are alive just before a_{j-1}, and D_j is the set of individuals i who die in interval I_j.

The DLFTM is a model for the functional form of $p_j(\mathbf{x})$. Specifically,

$$p_j(\mathbf{x}) = (1 + e^{\alpha_j + \mathbf{x}\boldsymbol{\beta}})^{-1}, \; 1 \le j \le k, \quad (3)$$

where $\boldsymbol{\beta} = (\beta_1, \ldots, \beta_m)^T$ is an $m \times 1$ vector of unknown regression coefficients relating the covariate vector $\mathbf{x} = (x_1, \ldots, x_m)$ to p_j, and the a_js are interval-specific parameters (i.e., the interval effects). Because (3) implies

$$\frac{1 - p_j(\mathbf{x} + \Delta)}{p_j(\mathbf{x} + \Delta)} = e^{\Delta\boldsymbol{\beta}} \left[\frac{1 - p_j(\mathbf{x})}{p_j(\mathbf{x})} \right], \; 1 \le j \le k \quad (4)$$

for any Δ, it follows that $\boldsymbol{\beta}$ relates the odds of death in any interval I_j for covariate value $\mathbf{x} + \Delta$ [i.e., the left side of (4)] to the odds of death in I_j for covariate value $\mathbf{x}$. Thus, if $\beta_r > 0$ (<0) for some $1 \le r \le m$, the odds of dying in interval I_j increases (decreases) as x_r increases. The parameter α_j is seen from (3) to equal $\ln\{[1 - p_j(0)]/p_j(0)\}$, the log-odds of death in interval I_j when $\mathbf{x} = 0$.

For a particular choice of the covariate vector $\mathbf{x}$ (i.e., including terms to represent treatments, dependence on SL, etc.), the unknown parameters $\boldsymbol{\beta}$ and $\boldsymbol{\alpha} = (\alpha_1, \ldots, \alpha_k)^T$ in the DLFTM (3) are estimated by substituting (3) into the likelihood (2), and then maximizing the resulting "constrained likelihood" (5) with respect to $\boldsymbol{\beta}$ and $\boldsymbol{\alpha}$ (Lawless 1982).

$$L(\boldsymbol{\alpha}, \boldsymbol{\beta}) = \prod_{j=1}^{k} \left[\left(\prod_{i \in D_j} \left\{ \frac{e^{\alpha_j + \mathbf{x}_i\boldsymbol{\beta}}}{1 + e^{\alpha_j + \mathbf{x}_i\boldsymbol{\beta}}} \right\} \right) \left(\prod_{i \in R_j - D_j} \{1 + e^{\alpha_j + \mathbf{x}_i\boldsymbol{\beta}}\}^{-1} \right) \right]. \quad (5)$$

The logarithm of this constrained likelihood is

$$\log L(\boldsymbol{\alpha}, \boldsymbol{\beta}) = \sum_{j=1}^{k} \left[\sum_{i \in D_j} (\alpha_j + \mathbf{x}_i\boldsymbol{\beta}) - \sum_{i \in R_j} \ln(1 + e^{\alpha_j + \mathbf{x}_i\boldsymbol{\beta}}) \right]. \quad (6)$$

The maximum likelihood estimators (mles) $\hat{\boldsymbol{\alpha}}$ and $\hat{\boldsymbol{\beta}}$ of $\boldsymbol{\alpha}$ and $\boldsymbol{\beta}$ are obtained by maximizing (6). The maximization is done by the Newton–Raphson algorithm, which iteratively solves the so-called likelihood equations (writing $\mathbf{x}_i = (x_{i1}, \ldots, x_{im})$)

$$\frac{\partial \log L}{\partial \beta_r} = \sum_{j=1}^{k} \left(\sum_{i \in D_j} x_{ir} - \sum_{i \in R_j} \frac{x_{ir} e^{\alpha_j + \mathbf{x}_i\boldsymbol{\beta}}}{1 + e^{\alpha_j + \mathbf{x}_i\boldsymbol{\beta}}} \right) = 0, \; r = 1, \ldots, m; \text{ and} \quad (7)$$

$$\frac{\partial \log L}{\partial \alpha_v} = \sum_{i \in D_v} (1) - \sum_{i \in R_v} \frac{e^{\alpha_v + \mathbf{x}_i\boldsymbol{\beta}}}{1 + e^{\alpha_v + \mathbf{x}_i\boldsymbol{\beta}}} = 0, \; v = 1, \ldots, k. \quad (8)$$

The interval-specific survival probability estimates, $\hat{p}_j(\mathbf{x})$ are obtained by substituting the *mle*s, $\hat{\boldsymbol{\alpha}}$ and $\hat{\boldsymbol{\beta}}$ into (3). The survival probability estimates $\hat{\underline{P}}_j(\mathbf{x})$ are then obtained by plugging the $\hat{p}_j(\mathbf{x})$ into (1). The issue of which variables to include in the regressor vector $\mathbf{x}$ (e.g., linear or quadratic functions of SL, treatment by SL interactions, etc.) was addressed by beginning with a model a priori deemed sufficiently flexible and then testing a sequence of nested models until reaching the most parsimonious model that adequately explained the data. Goodness-of-fit was assessed by comparing the most parsimonious fitted model to the a priori model (i.e., the model containing at most quadratic functions of SL and treatment by SL interactions) by a Wald statistic.

To conduct inferences using the estimated $\hat{\underline{P}}_j(\mathbf{x})$s, their covariance matrix was needed. This matrix was derived by a sequence of three steps, the first of which was to construct the covariance matrix of the estimators $\hat{\boldsymbol{\alpha}}$ and $\hat{\boldsymbol{\beta}}$. An estimate of the covariance matrix of $\hat{\boldsymbol{\theta}} = (\hat{\boldsymbol{\alpha}}, \hat{\boldsymbol{\beta}})$ was obtained as the negative of the inverse of the matrix of the second-order partial derivatives of $\log L$ (6) (i.e., $-H^{-1}$, where H is the Hessian matrix). The covariance matrices of the derived estimates $\hat{p}_j(\mathbf{x})$ and $\hat{\underline{P}}_j(\mathbf{x})$ were propagated, in turn, from the covariance matrix of $\hat{\boldsymbol{\theta}}$ by the Delta method (Serfling 1980). See Appendix A for details.

Estimating Median Survival Time

A unique aspect of our application of the DLFTM to analyze salinity as a lethal factor in *P. perna* was our incorporation of a method of estimating, and computing variances for, the covariate-adjusted median survival times. This method allows factorial-type comparisons of the covariate-adjusted medians across treatments. Median survival time, $M(\mathbf{x})$, which satisfies $S(M(\mathbf{x})|\mathbf{x}) = 0.5$, was estimated by assuming that the survival function, $S(t|\mathbf{x})$, was linear in t (for fixed $\mathbf{x}$) over each time interval $[a_{j-1}, a_j)$. Specifically, given that $\hat{\underline{P}}_j(\mathbf{x}) < 0.5 \le \hat{\underline{P}}_{j-1}(\mathbf{x})$ for some $1 \le j \le k$, then the median estimate is (using linear interpolation)

$$\hat{M}_j(\mathbf{x}) = a_j + \left[\frac{a_j - a_{j-1}}{\hat{P}_j(\mathbf{x}) - \hat{P}_{j-1}(\mathbf{x})} \right] \left[\frac{1}{2} - \hat{P}_j(\mathbf{x}) \right], \quad (9)$$

for the interval index j in which $\hat{P}_j(\mathbf{x}) < 0.5 \le \hat{P}_{j-1}(\mathbf{x})$. However, this interval is itself a random variable, so it is necessary to express the median estimate as

$$\hat{M}(\mathbf{x}) = \sum_{j=1}^{k} \hat{M}_j(\mathbf{x}) I[\hat{P}_j(\mathbf{x}) < 0.5 \le \hat{P}_{j-1}(\mathbf{x})] \quad (10)$$

where $\hat{M}_j(\mathbf{x})$ is given by (10), and the indicator variable [second factor in the summand in (10)] equals 1 if the parenthetical inequality holds and is zero otherwise [note that at most, one of the terms in the sum in (10) is nonzero]. In plain English, this just says to "find the interval j where the survival probabilities $\hat{\underline{P}}_j(\mathbf{x})$ cross 0.5 and use the estimate (9)." Equation (10) defines the estimated median $\hat{M}(\mathbf{x})$ only if $\hat{\underline{P}}_k(\mathbf{x}) \le 0.5$. Otherwise, $\hat{M}(\mathbf{x})$ is undefined.

To obtain $\text{var}[\hat{M}(\mathbf{x})]$ and $\text{cov}[\hat{M}(\mathbf{x}_1), \hat{M}(\mathbf{x}_2)]$ for $\mathbf{x}_1 \ne \mathbf{x}_2$, we again want to appeal to the delta-method, because, as is apparent from (10), $\hat{M}(\mathbf{x}) = G[\hat{\underline{P}}_1(\mathbf{x}), \ldots, \hat{\underline{P}}_k(\mathbf{x})]$ for an albeit complicated function G. However, this approach failed in this case, because the function G is not differentiable with respect to the $\hat{\underline{P}}_j$s [the indicator functions in (10) are discontinuous and, hence, not differentiable, so that the delta method does not apply]. To avoid this problem, we used an approximation method detailed in Appendix B.

By manipulating the regressor vector $\mathbf{x}$, estimates (and their covariance matrix) of covariate-adjusted median survival time were obtained for each treatment combination. Specifically, let M denote the vector of the true median survival times for the various treatments, and for target values of the continuous regressors (e.g., SL). Let $\hat{M}$ denote the corresponding vector of estimates, and let

$\text{Var}(\hat{M})$ denote the covariance matrix of $\hat{M}$ as obtained above. Comparison of median survival times across treatments was carried out via testing hypotheses of the form H_o: $\boldsymbol{CM} = 0$, where $\boldsymbol{C}$ is a hypothesis matrix of coefficients having linearly independent rows, using the Wald statistic

$$W = (\mathbf{C}\hat{\mathbf{M}})^T[\mathbf{C}Var(\hat{\mathbf{M}})\mathbf{C}^T]^{-1}(\mathbf{C}\hat{\mathbf{M}}). \quad (11)$$

Under H_o, the statistic W is distributed approximately chi-square with m degrees of freedom, where m is number of rows in $\mathbf{C}$. Large values of W provide evidence against H_o. Type I error was controlled using the Scheffé method for an experimentwise error rate of $\alpha = 0.05$.

RESULTS

Survivorship in *Perna perna* declined in hyposaline and hypersaline treatments. Salinities ranging from 15–50 ppt appeared nonlethal to this species, with at least 80% of individuals surviving the 30-day (720 h) exposure period. Following the 30-day period, surviving individuals were cut from byssal attachments and transferred back to full-strength seawater and allowed 72 h to reattach. Thereafter, the average number of attached byssal threads per individual and the associated salinity concentration were as follows: 15 (15 ppt), 11 (20 ppt), 13 (30 ppt), 13 (40 ppt), and 8 (50 ppt). The capacity for byssal reattachment indicated that individuals exposed to salinities of 15–50 ppt were not physiologically damaged. In contrast, complete sample mortality was observed at the following times and salinities: 360 h (0 ppt), 408 h (5 ppt), 648 h (10 ppt), 168 h (55 ppt), and 216 h (60 ppt) (Fig. 1).

In the most parsimonious, fitted survival model (Eq. 3), survival was significantly correlated to both SL and SL^2, but the SL effects did not interact with salinity treatment ($\chi^2_{(18)} = 18.7$; $P = 0.412$). Specifically,

$$\log\left[\frac{1 - p_j(\mathbf{x})}{p_j(\mathbf{x})}\right] = \beta_o + \sum_{T=1}^{9}\beta_T I_T + \gamma_1 SL + \gamma_2 SL^2$$

where β, γ_1 and γ_2 are coefficients for treatment and SL, respectively, and I_T is the indicator variable for salinity treatment T (i.e., $I_T = 1$ for salinity treatment T and zero otherwise).

The estimated survival functions [i.e., the linearly interpolated $\underline{\hat{P}}_j(\mathbf{x})$s] for lethal salinity treatments, adjusted to the sample mean SL (40 mm), indicated decreasing survivorship probabilities as salinity concentrations departed from full strength seawater (35 ppt) with those in the most saline treatments (i.e., 55 and 60 ppt) having the lowest median survival times (Fig. 1).

The relationship between survivorship and SL can be shown by using the model to estimate median survival at the 10th, 25th, 50th, 75th, and 90th percentiles of the SL distribution in each salinity treatment. This analysis indicated that intermediate-sized individuals (48 mm SL) had longer survival times (Fig. 2). For an average-sized individual, SL = 40 mm, the median survival times and associated salinity concentrations were as follows: 191 h (0 ppt),

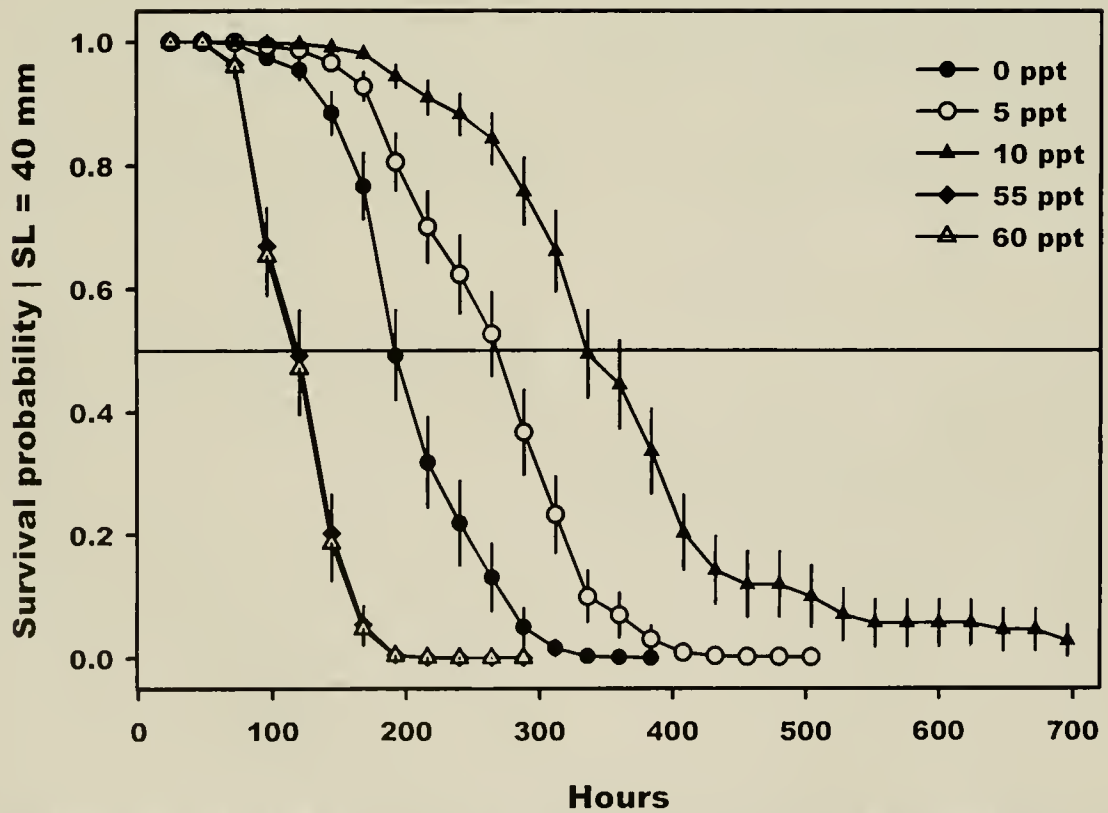

Figure 1. Survivorship curves for standard 40 mm shell length specimens of *Perna perna* chronically exposed to lethal salinity treatments. Lines represent linear interpolations of the cumulative survival probabilities, the $\underline{\hat{P}}_j$s, between successive 24-h sampling intervals in each salinity treatment. Error bars represent the standard errors of the $\underline{\hat{P}}_j$s. A median survival time is the hour at which the survival probability curve crosses the solid horizontal line at a survival probability of 0.5.

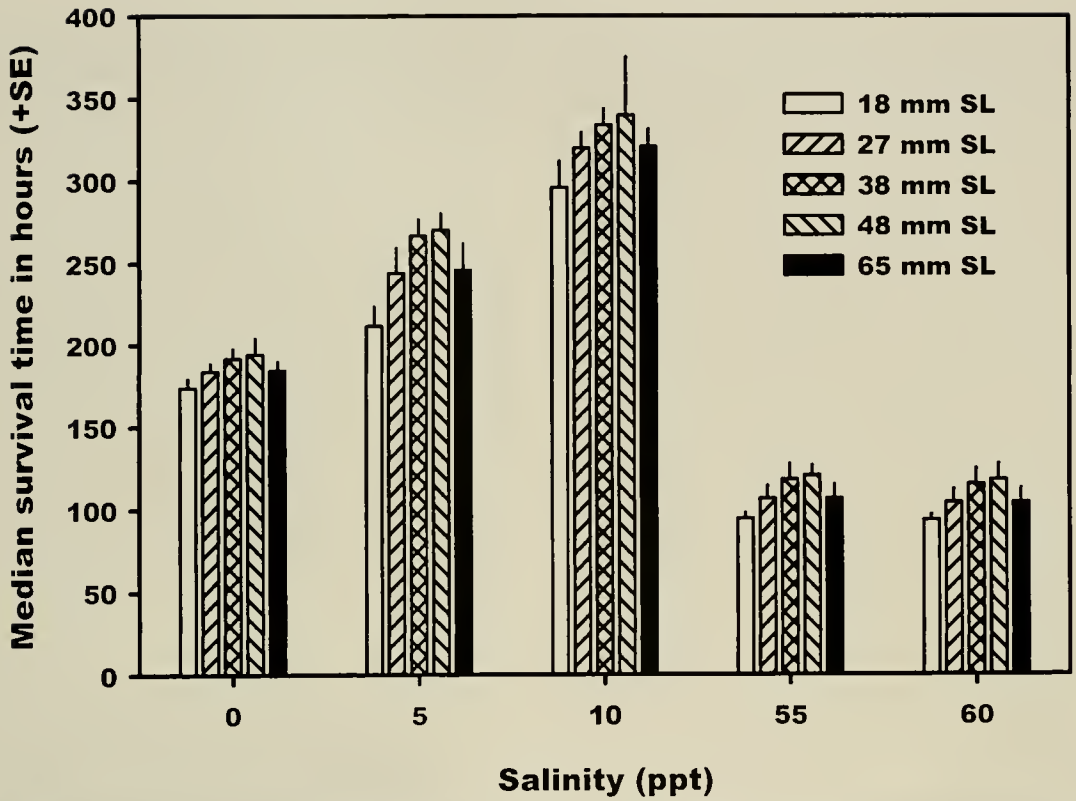

Figure 2. Median survival times (h) for Texas specimens of *Perna perna* in lethal salinity treatments adjusted to the 10th (18 mm SL), 25th (27 mm SL), 50th (38 mm SL), 75th (48 mm SL), and 90th (65 mm SL) percentile of the individual shell length (SL in mm) distribution. Error bars represent the standard errors of median survival estimates.

268 h (5 ppt), 335 h (10 ppt), 119 h (55 ppt), and 116 h (60 ppt) (Table 1).

Wald statistics for all-pairwise comparisons of lethal salinity treatment median survival times adjusted to the sample mean SL (40 mm) indicated significant treatment differences (Table 1). For the salinity treatments in which survival curves never fell below 0.5, median estimates could not be computed. However, because the odds ratio method still works, treatment survival distributions could be compared directly from their estimated treatment coefficients in the model (Eq. 3). For example, exponentiation of the coefficient for the nonlethal 15 ppt salinity treatment (Table 2), $e^{-6.002} = 0.0025$, indicates that the odds of death (in any time interval) in the 15 ppt salinity treatment were only about 0.25% ($\chi^2_{[9]} = 116.64$, $P < 0.0001$) of the odds of death in the 0 ppt salinity reference cell treatment (given SL = 40 mm). Pairwise testing of treatment coefficients indicated that the odds of death did not differ among the 15, 20, 30, 40, and 50 ppt salinity treatments whose survival probabilities never dropped below 0.5. The

TABLE 1.

Survivorship of standard 40 mm shell length (SL) Texas specimens of *Perna perna* exposed to lethal salinity treatments of 0, 5, 10, 55, and 60 ppt.

Salinity Treatment (ppt)	Median Survival Time (h ± SE)	Sample of 100% Mortality (h)	*n*	SL Range (mm)
0	191.36 (6.27)[a]	324	46	10.2–94.3
5	268.03 (9.94)[b]	372	44	10.9–77.5
10	335.17 (10.1)[c]	636	38	17.6–78.5
55	119.00 (9.68)[d]	156	45	12.8–87.2
60	116.22 (9.42)[d]	204	44	10.0–84.6

Median survival times with the same superscript are not different at $P < 0.05 \chi^2_{[5]}$

TABLE 2.

Estimated treatment coefficients ($\hat{\beta}$) and standard errors (SE) for Texas specimens of *Perna perna* relating the log-odds of death (in any time interval) to the 0 ppt salinity level reference cell treatment given shell length (SL) equals 40 mm.

Effects	$\hat{\beta}$	SE ($\hat{\beta}$)	Odds of Death $e^{(\beta)}$	SE (Odds of Death) $e^{(\beta)}SE(\hat{\beta})$
5 ppt salinity	−1.302	0.2897	0.2720	0.07879
10 ppt salinity	−2.672	0.3545	0.0691	0.02450
15 ppt salinity	−6.002	0.5557	0.0025	0.00137
20 ppt salinity	−8.194	1.0919	0.0003	0.00030
30 ppt salinity	−7.999	1.0895	0.0003	0.00037
40 ppt salinity	−8.012	1.0896	0.0003	0.00036
50 ppt salinity	−6.782	0.7200	0.0011	0.00082
55 ppt salinity	2.888	0.3757	17.9574	6.74658
60 ppt salinity	2.958	0.3788	19.2594	7.29547
SL	−0.222	0.0854	0.8009	0.06830
SL^2	0.311	0.0614	1.3648	0.08370

odds of death did differ between the 15–50 ppt salinity level treatments and the extreme hyper- (55–60 ppt) and hyposaline (0–10 ppt) treatments. Among the lethal treatments (i.e., 0–10 ppt and 55–60 ppt concentrations), the odds of death differed between all pairs, except the 55 and 60 ppt salinity concentrations, which is reasonable, considering their survival curves were very similar (Fig. 1).

In low-salinity treatments, mussels initially responded by closing the shell valves (Fig. 3). After 72, 48, 48, and 24 h, individuals were observed to open the shell valves in the 0, 5, 10, and 15 ppt salinity treatments. At the lowest salinity treatments (i.e., 0, 5, and 10 ppt), mantle tissues were observed to swell outside of the shell valves and may actually have forced them apart. Under the extreme hypersaline conditions (e.g., 55 and 60 ppt), individuals gaped widely from the onset of treatment until death. In nonlethal salinity treatments, individuals were open normally at the first 24-h observation period. However, there seemed to be a positive correlation between the percentage of open individuals and salinity in nonlethal treatments, with fewer individuals displaying open valves at progressively lower nonlethal salinity exposures (Fig. 3). The average percentages of open individuals in the 30-day observation period and their associated salinity concentrations were as follows: 17.2 ± 7.9 SD (15 ppt), 30.2 ± 7.5 SD (20 ppt), 37.1 ± 10.2 SD (30 ppt), 36.3 ± 8.2 SD (40 ppt), and 38.7 ± 9.6 SD (50 ppt).

DISCUSSION

In this study, we used a DLFTM to estimate covariate-adjusted median survival times based on interval-level data. We believe that for interval-level survival data, our strategy of using medians (where possible) to summarize survival experience is a helpful complement to the usual survival curves and odds ratios. Unlike analysis of covariance (ANCOVA) approaches, the DLFTM, like other regression-type models, can provide meaningful estimates of median survival time at fixed covariate values even in the presence of variable interactions.

Texas *P. perna* survived and maintained normal activity (e.g., maintained byssal thread production) over salinities of 15 to 50 ppt, a salinity tolerance range similar to that of 19–44 ppt deter-

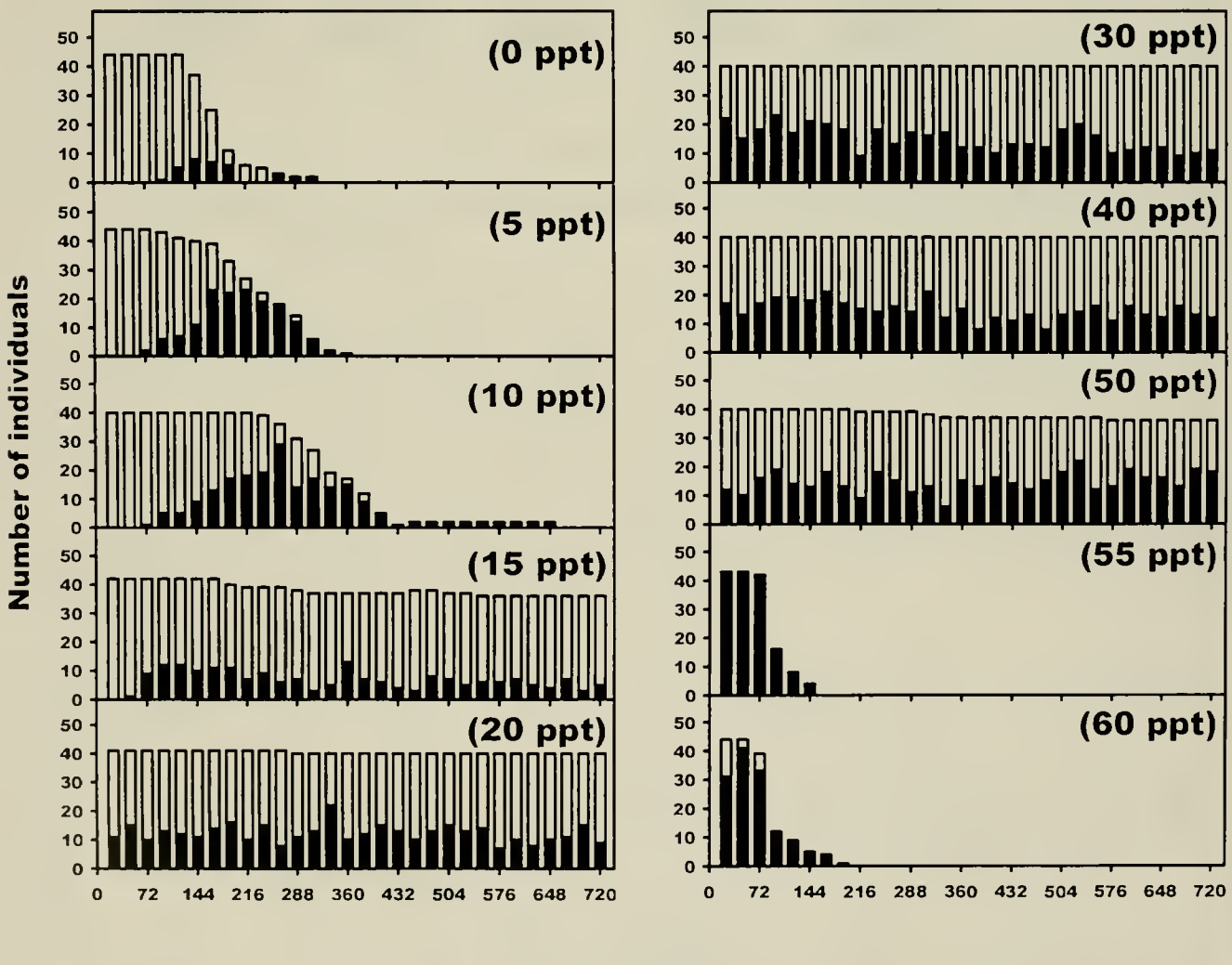

Figure 3. Shell valve closing behavior in Texas specimens of *Perna perna* exposed to chronic salinities ranging 0–60 ppt. Histogram bars represent numbers of living individuals and the solid portion of bars, the numbers of living individuals with shell valves open at each 24-h sampling interval.

mined for similar sized (45–50 mm SL) Brazilian *P. perna* (Salomão et al. 1980). A similar incipient low salinity value of 16 ppt was recorded for *Perna viridis* (L.) from India (Sundaram and Shafee 1989). Although the salinity tolerance we recorded for Texas *P. perna* generally agreed with data for South American specimens, recorded survival times were markedly different. At salinities of 4 and 9 ppt, complete sample mortality was observed after 102 hours in Brazilian specimens of *P. perna*. In contrast, Texas specimens of *P. perna* persisted for up to 360, 408, and 648 h in salinities of 0, 5, and 10 ppt, respectively. Similar to Brazilian data for *P. perna* salinity tolerance, complete sample mortality was observed to occur in individuals of *P. viridis* within 96 h upon exposure to salinities ranging 0–11 ppt (Sundaram and Shafee 1989). Median survival times reported for the Brazilian *P. perna* were 68, 49, 45, and 96 h on exposure to lethal salinities of 4, 9, 14, and 49 ppt, respectively (Salomão et al. 1980). In our study, median survival times were considerably greater than those of Brazilian specimens of *P. perna* or specimens of *P. viridis*.

Animal size was found to affect survival duration significantly in Texas specimens of *P. perna*. However, although the SL range of tested individuals was considerable (~15–70 mm SL), the range of median survival time in different sized individuals varied by only 22% or 59 h maximally. Although the largest individuals in the sample had reduced survival, the more general trend was increasing survival duration with increasing SL. This result generally agrees with that of Castagna and Chanley (1973) who demonstrated, among 29 tested marine bivalve species, that although smaller specimens generally succumbed more rapidly to lethal salinity exposures than did larger specimens, variation in individual size was not correlated with the minimum tolerated salinity.

Individuals responded to marked salinity reductions (15 ppt or ~43% dilution) by closing the shell valves. Shell valve closing behavior in bivalves allows survival of temporarily reduced salinities encountered in intertidal or estuarine environments during periods of heavy rainfall and/or freshwater run off (Gilles 1972, Davenport 1981, Akberali and Trueman 1985). Valve closure extended survival times in Brazilian specimens of *P. perna* relative to individuals with artificially propped open valves (Salomão et al. 1980). However, even when the valves are tightly closed, tissues are not completely isolated from the external environment. Thus, valve closing behavior provides protection from salinity stress only over relatively short periods. Indeed, even with the shell valves closed, *P. perna* tissues reach osmotic equilibrium within 72 h of exposure to salinities of 19–39 ppt (Salomão and Lunetta 1989). A similar value of 96 h has been reported for *Mytilus edulis* L. exposed to salinities ranging from 9–36 ppt (Gilles 1972).

The shell valve closing response is mediated by peripheral receptors located on the mantle margins and/or siphon surfaces (Davenport 1981, Berger and Kharazova 1997). These peripheral receptors are of two types: osmoreceptors, sensitive to osmotic pressure, and receptors sensitive to changes of specific ion concentrations (Berger and Kharazova 1997). Such receptors not only trigger valve closure in response to salinity change, but also enable detection of relative salinity concentrations. When transferred from full strength seawater (35 ppt) to lower salinities, the proportion of individuals of *P. perna* with closed valves was directly proportional to the seawater concentration: the lower the salinity, the greater the tendency to close the valves (Fig. 3). This result is consistent with the valve closure behavior in response to variable salinity recorded for other marine and estuarine bivalve species (Akberali 1978, Bailey et al. 1996).

Davenport (1981) found that increasing salinity is registered by special detectors located in the tentaculate portion of the inhalant siphon of *Mytilus edulis*. These receptors, requiring the presence of both sodium and magnesium ions, trigger shell valve parting at salinity concentrations similar to those that induce valve closure under declining salinity conditions (~ 17 ppt, Davenport 1981). It is unknown whether similar receptors are responsible for the gaping of individuals of *P. perna* observed in the highest salinity treatments (i.e., 55 and 60 ppt). Exposure to these elevated salinities seemed to have a narcotic effect on this species. Lack of an appropriate protective valve-closing response suggests that *P. perna* rarely encounters hyperosmotic conditions and is an indication that it is limited to marine intertidal and open-water estuarine habitats. Given that the tissues of specimens of *P. perna* will eventually come into osmotic equilibrium with their external environment, it seems that *P. perna* cannot tolerate haemolymph osmolarities of less than 430 mOsm (15 ppt) or greater than 1428 mOsm (50 ppt).

The range of salinity tolerated by marine bivalves in laboratory experiments is often a good predictor of their distribution in natural habitats (Castagna and Chanley 1973). Castagna and Chanley (1973) observed that the experimentally determined salinity tolerance of a number of marine bivalves adequately explained their distributions in nature. When a species' natural distribution did not include its entire tolerated salinity range, it was usually attributed to a species being near its geographic limit where other limiting factors, particularly temperature, became more important limiting factors. Within its endemic range, *P. perna* occurs primarily in open-water, high-energy hard-shore habitats where salinity remains relatively constant near full-strength seawater (Berry 1978). However, in Brazil, it is reported from semi-enclosed lagoons where salinities may be as low as 12.7 ppt (Marques et al. 1991). Although *P. perna* rarely occurs naturally in enclosed-estuarine habitats, it thrives when relocated to such habitats for culturing in South America and Sri Lanka (Indrasena and Wanninayake 1994). Indeed, some of the highest growth rates reported for this species are from culture grow-outs in lagoons where salinities ranged 20–45 ppt (Indrasena and Wanninayake 1994).

The salinity regimes of coastal environments vary temporally and spatially over short (tidal or storm induced) and long durations (seasonal influences). Bivalve salinity tolerance can be influenced by previous salinity experience (i.e., acclimation salinity), magnitude of salinity change, and/or rate of change (see Remane and Schlieper 1971 and Kinne 1971 for reviews). In this study, the incipient salinity limits of *P. perna* were determined by chronic exposures of up to 30 days following direct transfer from full-strength seawater (35 ppt) into one of 10 test salinities. Because of the long exposure periods involved, individuals had sufficient time to acclimate to test salinities near this species upper and lower incipient limits, allowing relatively accurate estimation of *P. perna*'s incipient salinity range. In addition, direct transfer to test salinities allowed evaluation of the colonization potential of *P. perna* after rapid, anthropomorphically mediated introduction of adults into new habitats. Pernids foul the hulls of international vessels (Carlton 1987). Thus, adult mussels byssally attached to anchor chains and/or the hulls of transoceanic vessels and barges can be transferred directly into a new habitat whose salinity regime varies from that of the source population. Our data suggest that if other environmental conditions are favorable, anthropomorphically introduced *P. perna* could colonize waters within a salinity range of 15–50 ppt.

The incipient upper and lower salinity limits determined herein indicate that *P. perna* could potentially colonize areas in the Gulf of Mexico outside of its present Texas/Mexico range (Hicks and Tunnell 1993, Hicks and Tunnell 1995). The 15–50 ppt tolerated salinity range of Texas *P. perna* suggests that this species could potentially invade most Gulf of Mexico coastal habitats, including those from which raw water is drawn by industrial and power generation facilities, particularly in the Houston ship channel and Mobile Bay; potentially making them susceptible to the macrofouling reported for this species in India (Ragapogal et al. 1995). Thus, the dispersal of this invasive nonindigenous species should continue to be closely monitored in the North American Gulf of Mexico and southern North Atlantic coastal habitats.

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APPENDIX 1: EXAMPLE OF THE DELTA METHOD

The delta method is a general technique of variance propagation as follows. Let $\boldsymbol{U}$ be a p × 1 random vector that is distributed approximately multivariate normal (MVN) with mean E$\boldsymbol{U}$ and covariance matrix Var ($\boldsymbol{U}$). Let $f(z)$ be an $s \times 1$ vector function of p variables; i.e.,

$$f(z) = \begin{bmatrix} f_1(z) \\ \cdot \\ \cdot \\ \cdot \\ f_s(z) \end{bmatrix}.$$

Let J_f denote the $s \times p$ Jacobian matrix of f whose (a, b) entry is $\partial f_a/\partial z_b$. The delta method then asserts that the $s \times 1$ random vector $f(\boldsymbol{U})$ is also approximately MVN, with mean

$$E\{f(\boldsymbol{U})\} \approx f(E\boldsymbol{U}) \text{ and covariance matrix}$$

$$\text{Var}\{f(\boldsymbol{U})\} \approx J_f(E\boldsymbol{U})\{\text{Var}(U)\}\boldsymbol{J}_f^T(E\boldsymbol{U}).$$

As an illustration of the delta method in our problem, we give the details of obtaining the covariance matrix of the interval-specific estimates $\hat{\boldsymbol{p}} \equiv [\hat{p}_1(\boldsymbol{x}), \ldots, \hat{p}_k(\boldsymbol{x})]^T$ from the covariance matrix of $\hat{\theta}$ (Here $\boldsymbol{x}$ is specified and fixed). The computer program performs these calculations, as well as those for obtaining the covariance matrix of the $\hat{\underline{P}}_j$s from the $\hat{p}_j$s. Because $\hat{p}_j(\mathbf{x})$ is obtained by plugging $\hat{\theta} = (\hat{\alpha}, \hat{\beta})$ into (3), the vector $\hat{\boldsymbol{p}}$ is given by $\hat{\boldsymbol{p}} = \mathbf{f}(\hat{\theta})$, where

$$f(z) = \begin{bmatrix} f_1(z) \\ \cdot \\ \cdot \\ \cdot \\ f_k(z) \end{bmatrix}$$

and for $k \times 1$ vector $\boldsymbol{w} = (w_1, \ldots, w_k)^T$, $m \times 1$ vector $\boldsymbol{v} = (v_1, \ldots, v_m)^T$ and $\mathbf{z} = (\mathbf{w}, \boldsymbol{v})$,

$$f_j(\mathbf{z}) = (1 + e^{w_j + \mathbf{x}\boldsymbol{v}})^{-1}, \; 1 \le j \le k. \tag{12}$$

Now, because $\hat{\theta}$ is a maximum likelihood estimator, by general likelihood theory (see Serfling 1980) it holds that $\hat{\theta}$ is approximately MVN with mean $\boldsymbol{\theta} = (\boldsymbol{\alpha}, \boldsymbol{\beta})$ [i.e., the true values assumed to have generated the $\mathbf{G}_i$s via (3)] and covariance matrix (as noted above) $-\mathbf{H}^{-1}$. We applied the delta method to $\mathbf{U} = \hat{\theta}$, $E\mathbf{U} = \theta$, $\text{Var}(\mathbf{U}) = -\mathbf{H}^{-1}$, and the function $\mathbf{f}$ in (12) to get that $\hat{\boldsymbol{p}} = \mathbf{f}(\hat{\theta})$ is approximately MVN with $E(\hat{\boldsymbol{p}}) \approx \mathbf{f}(\theta) = \boldsymbol{p}(\mathbf{x}) = [p_i(\mathbf{x}), \ldots, p_k(\mathbf{x})]^T$ (the true value) and $\text{Var}(\hat{\boldsymbol{p}}) \approx \mathbf{J}_f(\hat{\theta})\{-\mathbf{H}^{-1}\}\mathbf{J}_f(\hat{\theta})$, where the $k \times (k + m)$ Jacobian $J_f(\mathbf{z})$ is given by

$$\mathbf{J}_f(\mathbf{z}) = \begin{bmatrix} \frac{\partial f_1}{\partial w_1} & \cdots & \frac{\partial f_1}{\partial w_k} & \frac{\partial f_1}{\partial v_1} & \cdots & \frac{\partial f_1}{\partial v_m} \\ \cdot & & & & & \\ \cdot & & & & & \\ \cdot & & & & & \\ \frac{\partial f_k}{\partial w_1} & \cdots & \frac{\partial f_k}{\partial w_k} & \frac{\partial f_k}{\partial v_1} & \cdots & \frac{\partial f_k}{\partial v_m} \end{bmatrix}$$

Specifically, for any $1 \le j \le k$ and $1 \le r \le k$,

$$\frac{\partial f_j}{\partial w_r} = \frac{\partial}{\partial w_r}\{(1 + e^{w_j + \mathbf{x}\boldsymbol{v}})^{-1}\} + \begin{cases} 0, & r \ne j \\ \dfrac{-e^{w_j + \mathbf{x}\boldsymbol{v}}}{(1 + e^{w_j + \mathbf{x}\boldsymbol{v}})^2}, & r = j \end{cases}$$

and for $1 \le s \le m$,

$$\frac{\partial f_j}{\partial v_s} = \frac{\partial}{\partial v_s}\{(1 + e^{w_j + x_1 v_1 + \cdots + x_m v_m})^{-1}\} = \frac{-x_s}{(1 + e^{w_j + \mathbf{x}\boldsymbol{v}})^2}.$$

APPENDIX 2: CALCULATION OF VARIANCE, VAR[$\hat{M}(\mathbf{x})$] AND COVARIANCE, COV[$\hat{M}(\mathbf{x}_1)$, $\hat{M}(\mathbf{x}_2)$] OF SL ADJUSTED MEDIAN SURVIVAL TIMES

Writing $z_j = \hat{\underline{P}}_j(\mathbf{x})$ to reduce notation, and writing $M_j(\mathbf{x}) = f_j(\mathbf{z})$, where $\mathbf{z} = (z_1, \ldots, z_k)$, $\hat{M}(\mathbf{x})$ can be written as $f(\mathbf{z})$, where

$$f(\mathbf{z}) = \sum_{j=1}^{k} f_j(\mathbf{z}) I(z_j < 0.5 \le z_{j-1}). \tag{13}$$

Now the indicator function (the source of the differentiability problem) is

$$I(z_j < 0.5 \le z_{j-1}) = I(z_{j-1} \ge 0.5)\, I(z_j < 0.5) = h(z_{j-1})[1 - h(z_j)] \tag{14}$$

where

$$h(t) = I(t \ge 0.5) = \begin{cases} 0, & t < 0.5 \\ 1, & t \ge 0.5 \end{cases}.$$

But $h(t)$ can be closely approximated (as ε approaches zero) by the function $h_\varepsilon(t) = \Phi(t - 0.5/\varepsilon)$, which is the cdf of the $N(0.5, \varepsilon^2)$ distribution (Fig. 4). In fact, $h_\varepsilon(t) \to h(t)$ as $\varepsilon \to 0$ for every $t \in [0,1] - \{0.5\}$. Furthermore, $h_\varepsilon(t)$ is, unlike $h(t)$, differentiable. Thus, we may closely approximate $I(z_j < 0.5 \le z_{j-1}) \approx h_\varepsilon(z_{j-1})[1 - h_\varepsilon(z_j)]$, and, hence, approximate $f(\mathbf{z})$ by the differentiable function $f_\varepsilon(\mathbf{z})$ for small ε:

$$f(\mathbf{z}) \approx f_\varepsilon(\mathbf{z}) \equiv \sum_{j=1}^{k} f_j(\mathbf{z}) h_\varepsilon(z_{j-1})[1 - h_\varepsilon(z_j)]. \tag{15}$$

Thus, we approximated the estimate $\hat{M}(\mathbf{x}) \equiv f(\hat{\underline{P}}_1, \ldots, \hat{\underline{P}}_k)$ by $f_\varepsilon(\hat{\underline{P}}_1, \ldots, \hat{\underline{P}}_k)$, to which the delta method applies. Details of this analysis appear in the computer code available from the authors upon request.

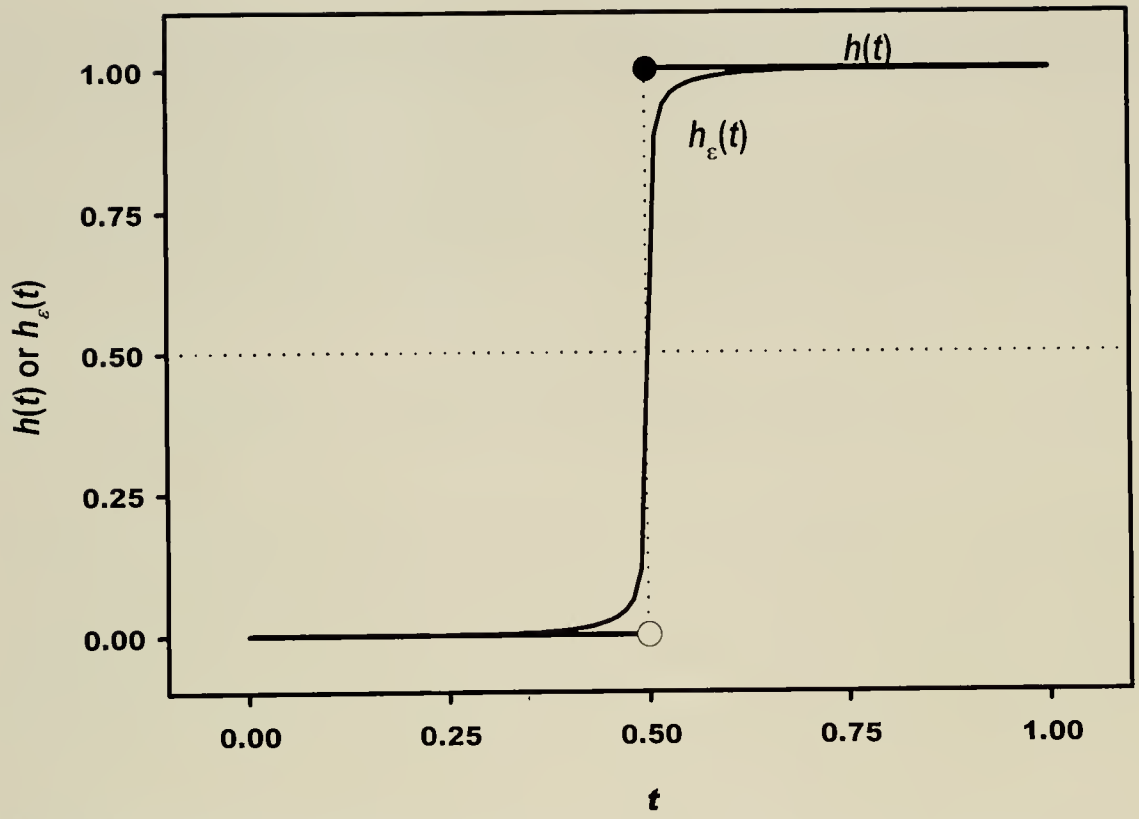

Figure 4. Illustration depicting the approximation of the indicator function $h(t)$ by the cdf of the $N(0.5, \varepsilon^2)$ distribution, $h_\varepsilon(t)$, as $\varepsilon \to 0$.

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A POLYMERASE CHAIN REACTION ASSAY FOR THE DETECTION OF GENOMIC DNA OF A RICKETTSIALES-LIKE PROKARYOTE ASSOCIATED WITH WITHERING SYNDROME IN CALIFORNIA ABALONE

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ABSTRACT The 16S rDNA from a Rickettsiales-like procaryote (RLP) infecting postesophageal tissues of black abalone *Haliotis cracherodii* Leach exhibiting signs of withering syndrome (WS) was amplified, cloned, and sequenced. The 16S rDNA sequence for the RLP was similar to that of species found in the genera *Ehrlichia, Anaplasma,* and *Wolbachia.* A polymerase chain reaction (PCR) test was developed that specifically amplifies a 160 bp segment of the 16S rDNA from the RLP associated with WS. Positive reactions were obtained for all black abalone samples of digestive gland or postesophagus known to be infected with the RLP by microscopic examinations of stained tissue sections. The PCR worked equally well for infected tissues of black and red abalone *H. rufescens* Leach. There was no amplification of genomic DNA from four other microbial species isolated from cultures of intestinal flora of abalone or from abalone deemed free of the RLP by microscopic examinations. This PCR test greatly increases the ability to detect the bacterium, because to date no means to grow the organism from marine invertebrates on synthetic media or in cell lines have been developed. This PCR test should allow detection of the RLP before the onset of clinical signs of withering syndrome in cultured or wild abalone stocks. Furthermore, the test may be useful in identifying reservoirs or other related RLPs in other marine invertebrates.

KEY WORDS: Polymerase chain reaction, ribosomal DNA, Rickettsiales, abalone, *Ehrlichia, Haliotis*

INTRODUCTION

The black abalone *Haliotis cracherodii* Leach, a species once common along much of the intertidal zone of southern California's rocky beaches, has suffered severe negative impacts because of the disease termed withering syndrome (WS) (Alstatt et al. 1996., Friedman et al., 1997, Haaker et al. 1992, Tissot et al. 1991, VanBlaricom et al., 1993, 1996). Affected populations of black abalone have been reduced to 1–10% of population densities observed before the onset of WS (Haaker et al. 1992). The disease has now been observed among farmed red abalone (*H. rufescens*) (Moore et al. in press), and this has prompted the California Department of Fish and Game to place a partial ban on movement of cultured red abalone from locations where WS is endemic to locations free of this disease. The epizootic manner in which the disease has spread throughout black abalone in the Channel Islands and the mainland indicate a role for an infectious etiologic agent (Lafferty and Kuris 1993). Although not completely proved, evidence suggests that WS is caused by a previously undescribed Rickettsiales-like prokaryote (RLP), which infects gastrointestinal epithelia (Friedman et al. 1997, Gardner et al. 1995). Withering syndrome and the associated RLP have also been documented in wild and cultured red abalone, *Haliotis rufescens* Leach, (Haaker et al. 1992, Moore et al. in press). Because of the inability to culture most marine RLPs, differentiation of these organisms is difficult and is based upon morphological characteristics using light and electron microscopy. Sensitive and accurate detection of the WS-associated RLP is critical to our understanding and control of the spread of WS. The goal of the current study was to develop a polymerase chain reaction (PCR) test to improve our detection and understanding of the biology of the RLP causing WS in abalone and other as yet unidentified hosts.

MATERIALS AND METHODS

DNA Extractions, Sequencing, and Alignments of rDNA

DNA isolation from whole tissue and from bacterial cells was performed as described by Sambrook et al. (1989) and Friedman et al. (in press). Briefly, rinsed tissues were homogenized in lysis/proteinase K buffer. After 1 h at 55 °C, the DNA was extracted in a phenol-chloroform solution. Isoamyl alcohol was added, mixed for 10 min, and centrifuged. The top aqueous phase was removed and 1/10 volume of 3 M sodium acetate was added. Cold absolute ethanol was added to precipitate the DNA. The pellet was washed in 70 % ethanol, air-dried, and resuspended in Tris-EDTA (TE) buffer. Alternatively, DNA was prepared using the QIAmp Tissue Kit (Qiagen Inc., Valencia, CA) following the manufacturers "mouse tail protocol". Bacterial 16S rDNA from infected abalone tissue was amplified using EUB A and EUB B universal eubacterial 16S rDNA primers (Giovannoni 1991). The RLP 16S rDNA gene was cloned into pCR2.1 using a TOPO Cloning Kit (Invitrogen, San Diego, CA) following manufacturer's protocols. Clones were screened using PCR with primers that flanked the multicloning site of the vector. Positive clones were selected from among those that had an insert of the appropriate size (~1550 bp). The 16S rDNA sequencing and alignments of sequences were performed as described previously (Andree et al. 1997). The completed sequence was used in a BLAST search of GenBank to confirm the similarity of the sequence to other Rickettsiales. The species appearing in the results of the BLAST search were compared with other closely related bacterial species in a pairwise analysis of sequence similarity (Table 1) in addition to a phylogenetic distance analysis (Friedman et al. in press).

TABLE 1.

A pairwise comparison of the 16S ribosomal DNA sequence similarity seen among closely related species.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1 WFS-RLP		74.0	73.3	73.2	77.3	75.4	75.5	75.8	71.3	69.7	73.4	73.3	70.9	69.8	69.0
2 *W. pipientis*			77.8	77.6	80.7	80.5	81.5	81.0	74.1	73.6	77.3	77.5	73.7	73.3	73.0
3 *E. sennetsu*				94.5	78.4	77.9	79.7	78.9	74.7	73.5	76.3	76.6	74.2	72.6	71.3
4 *E. risticii*					78.2	77.5	79.5	78.7	74.9	73.5	76.0	76.4	74.3	73.3	71.0
5 *A. marginale*						85.8	89.4	88.2	76.9	75.1	77.1	77.6	75.9	73.6	73.7
6 *C. ruminantium*							86.2	85.4	76.7	73.8	77.5	77.8	74.1	72.7	71.5
7 *E. phagocytophila*								91.2	76.8	74.3	78.3	78.5	75.7	74.0	73.1
8 *E. bovis*									76.1	74.1	77.6	77.6	75.6	73.2	72.9
9 *C. caryophila*										74.5	77.3	77.8	78.8	74.6	72.3
10 *C. burnettii*											74.4	75.0	72.5	80.5	78.1
11 *R. prowesekii*												93.0	76.7	74.3	71.8
12 *R. rickettsii*													77.1	74.3	71.8
13 NHP														72.3	70.3
14 *P. salmonis*															81.5
15 *E. coli*															

Primer Selection and Oligonucleotide Synthesis

A BLAST search of GenBank indicated three species, *Anaplasma marginales, Ehrlichia bovis*, and *Wolbachia pipientis*, were most similar to the 16S rDNA sequence amplified from infected abalone (clone designation p16RK3; GenBank accession number: AF133090). The four sequences above and that of *Piscirickettsia salmonis* and a Rickettsiales-like prokaryote from shrimp (Frelier et al. 1993) were aligned to identify those sequences most unique to the RLP from infected abalone. Several primers were selected for synthesis and testing. In an initial trial, two primers designated RA5-1 [$^{5'}$GTTGAACGTGCCTTCAGTTTAC$^{3'}$] and RA3-1 [$^{5'}$CTGAGGCCATCTGTTAAAATGG$^{3'}$], were synthesized (Gibco BRL, Inc., Gaithersburg, MD) and used in an initial screening of samples containing enriched RLP from abalone tissues. The best results were obtained using these primers in conjunction with an annealing temperature of 55 °C. An amplified product of 946 bp was obtained from all RLP-enriched samples tested (data not shown). Subsequent tests with DNA samples extracted from digestive gland tissues of diseased animals showed a poor correlation with the histology results (e.g., samples from known positive abalone were negative by PCR). We speculated that the 16S rDNA of the normal gut flora could be hybridizing with the primers for the PCR, reducing efficiency of amplification. Accordingly, we designed new primers for the WS-PCR based on a second alignment using the 16S rDNA sequences from p16RK3, *E. bovis, A. marginales, W. pipientis, P. salmonis*, and *E. coli* as a representative of intestinal flora. This alignment showed the sequence of primer RA3-1 to be completely conserved in the 16S rDNA of *E. coli*, and this could have been contributing to the poor results observed in the PCR by nonspecific hybridization of this primer to bacterial DNA in the sample. We, therefore, designed three additional primers designated RA5-6 [$^{5'}$GAAGCAATATTGTGAGATAAAGCA$^{3'}$], RA3-6 [$^{5'}$ACTTGGACTCATTCAAAAGCGGA$^{3'}$], RA3-8 [$^{5'}$CCACTGTGAGTGGTTATCTCCTG$^{3'}$] for testing as potential primers for the WS-PCR. The primers RA5-1 and RA3-6 were designed to amplify a sequence of ~160 bp from the 5′ end of the 16S rDNA (Fig. 1). The primer pair RA5-6 and RA3-8 was designed to amplify a sequence of ~230 bp from the 3′ end of the 16S rDNA (Fig. 1). The previously extracted DNA samples were then retested with these new primer sets.

We also examined the possibility of using crude cell lysates rather than purified DNA in the assays for the WS-PCR. Tissue samples from 26 abalone were prepared by homogenization in TE buffer and boiling for 5 minutes at 100 °C. From this solution, 3 µl was added to a PCR cocktail. After amplification, DNA was separated on 1.5 % agarose gels.

PCR Amplification of rDNA

All amplifications were performed in standard 50 µl reactions containing 10 mM Tris-HCl pH 8.3 (at 25 °C), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001 % w/v gelatin, 400 µM dNTPs, 5 µM tetramethyl ammonium chloride, 40 pmoles of each primer, and 2 U Taq polymerase. The PCR thermal cycler used was a model PTC-200 (MJ Research, Watertown, MA).

A 160 bp fragment (using primers RA5-1 and RA3-6) of the 16S rDNA from the RLP was amplified using 40 cycles of 1 min at 95 °C, followed by 30 sec at 50 °C, followed by 30 sec at 72 °C. The amplification cycles were preceded by a denaturation step, where samples were held at 95 °C for 5 min and followed by an extended elongation step where samples were held at 72 °C for 10 min.

Specificity of the PCR

Adult black abalone were collected from the Vandenberg Air Force Base, CA (in July 1996) where WS is epidemic and from Monterey, CA (in December 1998) where the RLP has been recently detected but where no signs of clinical WS have yet become apparent (Finley and Friedman, unpubl. obs). Additional samples of adult abalone were collected from the following two locations where neither WS nor the RLP had been observed: black abalone from Carmel Point in August 1997 and red abalone from Shelter Cove in December 1998. Farmed red abalone obtained in January 1999 from a facility in Central California were also examined. Visual assessment of WS was determined according to Friedman et al. (1997). Digestive gland, postesophagus and/or epipodium were collected and stored at −80 °C until DNA extraction. The hemolymph of abalone contains no blood-clotting factor; there-

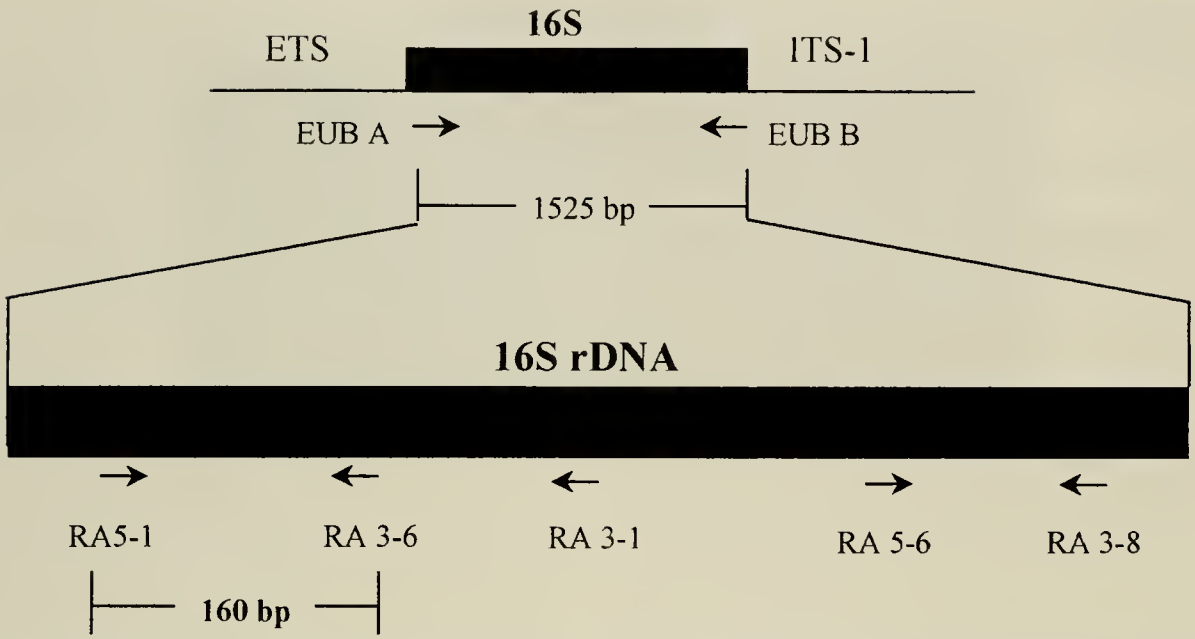

Figure. 1. Diagrammatic representation of the approximate location and orientation of primers used for development of the PCR assay to detect the Rickettsial-like prokaryote among abalone with withering syndrome (WS). Primers EUB A and EUB B were used in the initial amplification of the 16S rDNA from infected tissues.

fore, collection of samples from internal organs is fatal for the animal. DNA samples obtained from different abalone tissues were tested to identify those best for use in PCR assays and to determine if the RLP could be detected in nonlethal samples of the epipodium.

Genomic DNA of endogenous gut flora was tested for possible nonspecific amplification of DNA using the WS diagnostic primers. To obtain cultures for DNA extraction, postesophagus tissue (0.8–2.0gm) was dissected from three black abalone (Vandenberg Air Force Base, CA) and held separately in sterile vials containing 10 mL of 0.2 μm-filtered seawater, on ice. Vials were shaken vigorously to dislodge and suspend bacterial flora associated with epithelial surfaces, and the tissue was removed from each vial. The bacterial suspensions were spread onto marine and TCBS agar plates that were held at 15 °C. Colonies appearing after 7 days were streaked on marine agar plates. Four colonies from the TCBS plates and two from the marine agar plates were selected and passed several times on marine agar. Based on biochemical (API NFT Biomerieux Vitek, Hazelwood, MO) tests, supplemental tests, and colony morphology on TCBS and marine agar, four distinct isolates were obtained (Table 2). Cultures grown in TSB broth (supplemented with 2 % additional NaCl) were centrifuged (3,200 *g*, 10 min, 4 °C), resuspended in TE buffer (10 mM Tris-HCl, pH 7.5, 0.1 mM EDTA), and frozen at −80 °C. The presence of any RLPs among the colonies tested was precluded by the multiple passages of the isolates on artificial media. DNA was extracted from the bacterial cultures, as described above. Approximately 150 ng of genomic DNA from each of these isolates was tested with the primers RA5-1 and RA3-6.

Histology

Several 3-mm cross sections that included mantle, epipodium, postesophagus, digestive gland, and foot muscle were excised from each abalone, placed in Invertebrate Davidson's solution (Shaw and Battle 1957) for 24 h and processed for routine paraffin histology. Deparaffinized 5 μm tissue sections were stained with Harris's hematoxylin and eosin (Luna 1968) and assessed for the presence of RLPs, and condition of the foot muscle and digestive gland were evaluated (Friedman et al. 1997). The digestive gland was scored as 0 if normal, with terminal acini occupying most of the tissue present. A score of 1 represented a moderate degeneration of or replacement of terminal acini with transport ducts or connective tissues: whereas, a score of 2 represented a severe loss of acini. Similarly, condition of the foot muscle was scored as 0 when muscle bundles were tightly packed, 1 when a moderate loss

TABLE 2.

Characteristics of four postesophagous bacterial isolates grown in culture and tested for reactivity using the WS-RLP PCR protocol.

Isolate	Gram	Shape	Sucrose Reaction on TCBS	O/129 Sensitivity	H2S Production	PCR Reaction
150-A	Negative	Rods	Positive	Sensitive	Negative	Negative
150-B	Negative	Rods	Negative	Sensitive	Negative	Negative
213	Negative	Rods	Negative	Resistant	Positive	Negative
239	Negative	Rods	Negative	Resistant	Negative	Negative

of muscle bundles and concomitant increase in connective tissue was observed, and 2 when such loss was severe.

RESULTS

PCR Detection of the Putative Etiologic Agent of Withering Syndrome

The DNA from tissues of abalone with naturally acquired infections was screened for the presence of RLP DNA. Tissues from abalone collected from geographic regions where signs of WS and the associated RLP were absent served as negative controls. In addition, epipodial tissue was tested to determine if nonlethal samples could serve as diagnostic material for PCR analysis.

In total, we examined 23 animals by PCR (Table 3). We compared epipodium, digestive gland, and/or postesophagus for some individual animals. The 160 bp amplicon was present only in tissues from those animals that came from WS enzootic areas (most of which had proved to be positive by microscopic examination). The identity of the amplified DNA was confirmed by automated sequencing (data not shown). In addition, the identity of the species being detected was confirmed by *in situ* hybridization experiments in which the PCR primers were used as probes (Antonio et. al. in press). The yield of amplified DNA was greatest from postesophageal tissue (Fig. 2). This agrees with microscopic observations that demonstrated the postesophagus was more highly infected than the digestive gland. A weak amplification of DNA was obtained from some samples of epipodium from a subset of animals that tested strongly positive using the digestive gland.

Specificity of the PCR

Of the primer pairs tested, RA5-1 and RA3-6 gave the best results at an annealing temperature of 50 °C. Retesting of samples with this new primer produced a 160 bp amplicon from all tissues known to be positive for the RLP by microscopic examinations. In addition, all samples considered free of the RLP by microscopic examination were negative by PCR. There was no amplification of genomic DNA from the selected bacterial isolates from the intestine of black abalone or from a recently isolated *Piscirickettsia*-like organism isolated from white sea bass *Atractoscion nobilis* Ayres in California (unpubl. obs.). Only DNA extracted from abalone tissues known to contain RLP yielded a 160 bp amplicon. All samples of postesophagus and digestive gland from RLP-infected red and black abalone tested positive. There was a 250 bp amplicon present from the epipodial tissue of six black abalone collected from Vandenberg, Monterey, and Shelter Cove. However, two of these six samples yielded both amplicons (160 bp and 250 bp). Those with only the 250 bp amplicon were scored as negative based on the difference in the molecular weight and sequencing of the larger amplicon that indicated it was not related to bacterial 16S rDNA.

DISCUSSION

Diagnosis of infectious disease during the past century has generally relied on such methods as culture, direct observation of parasites or, more recently, antigen-based assays (Sethi et al. 1996). These methods may involve expense and time for sample

TABLE 3.

Detection of a Rickettsiales-like prokaryote by PCR and histology in digestive gland, postesophagous and epipodium tissues in black and red abalone in various stages of withering syndrome.

Animal #	Species	Source/Date	WS sign[a]	Digestive gland PCR[b]	Digestive gland Histology[c]	Postesophagus PCR[b]	Postesophagus Histology[c]	Epipodium PCR[b]	Epipodium Histology
1	Black	Carmel Pt. 8-97	0	–	0	nd	0	–	–
2	Black	Carmel Pt. 8-97	0	–	0	nd	0	–	–
3	Black	Carmel Pt. 8-97	0	–	0	nd	0	–	–
4	Black	Carmel Pt. 8-97	0	–	0	nd	0	–	–
5	Red	Monterey 12-98	0	–	0	nd	0	–	–
6	Red	Monterey 12-98	0	–	0	nd	0	–	–
7	Red	Monterey 12-98	0	–	0	nd	0	–	–
8	Red	Shelter Cove 12-98	0	–	0	nd	0	–	–
9	Red	Shelter Cove 12-98	0	–	0	nd	0	–	–
10	Red	Shelter Cove 12-98	0	–	0	nd	0	–	–
11	Black	Monterey 12-98	0	++	1	nd	1	+	–
12	Black	Monterey 12-98	0	++	1	nd	1	–	–
13	Black	Monterey 12-98	0	++	2	nd	2	–	–
14	Black	Vandenberg 1997	0	++	1	nd	2	–	–
15	Black	Vandenberg 1997	1	+	0	nd	2	+	–
16	Black	Vandenberg 1997	2	++	2	nd	3	+	–
17	Black	Vandenberg 1997	2	+++	3	nd	3	+	–
18	Red	Farm A 1-99	2	+++	2	nd	2	–	–
19	Black	Vandenberg 1997	3	–	0	+++	3	nd	–
20	Black	Vandenberg 1997	3	++	3	+++	3	nd	–
21	Black	Vandenberg 1997	3	+++	3	nd	3	++	–
22	Red	Farm A 1-99	3	+++	2	++++	3	–	–
23	Red	Farm A 1-99	3	+++	3	nd	2	–	–

[a] WE sign: Degree of body mass shrinkage (0 = within normal range, 1, 2, 3 = slighily, moderately, severely shrunken).

[b] Relative intensity of 160 bp amplicon band in ethidium bromide stained gels (– = absent, ++++ = brightest, nd = no data).

[c] RLP infection intensity by microscopic examination (0 = absent, 1 = low density, 2 = moderate, 3 = high).

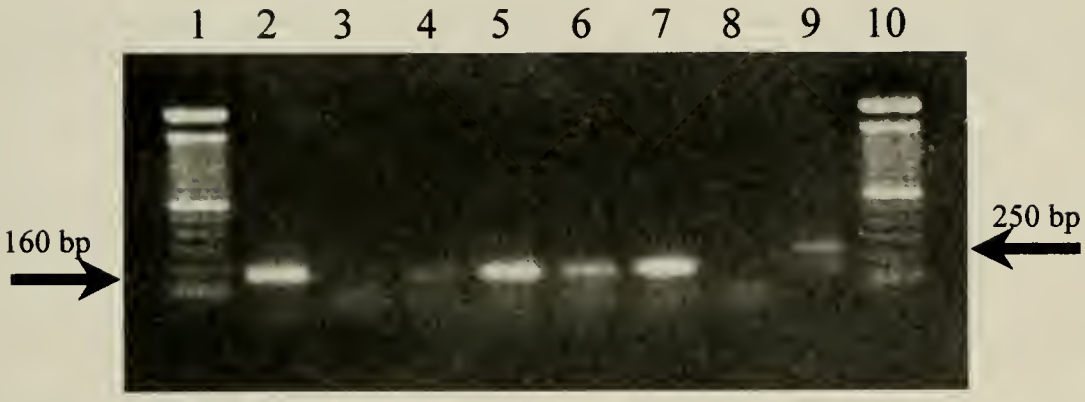

Figure. 2. Detection of a Rickettsial-like prokaryote in tissues from abalone with withering syndrome (WS) using a newly developed PCR assay. Approximately 300 ng of genomic DNA was used for each sample assay. Lanes 1and 10: 100 bp ladder molecular weight standard; lane 2: positive control sample (10 ng of plasmid p16RK3 containing cloned 16S rDNA); lane 3: negative control sample (uninfected black abalone postesophagus tissue); lanes 4 and 5, respectively: digestive gland and postesophagus of infected black abalone; lanes 6 and 7, respectively: digestive gland and postesophagus of infected red abalone; lane 8: negative control sample (150 ng of genomic DNA from prokaryote cultured from the gut of abalone); lane 9: epipodial tissue from infected black abalone displaying the 250 bp amplicon.

preparation or may lack sensitivity in detecting low numbers of parasites. Furthermore, many parasites, including the RLP from abalone, have not been cultured outside the host animal (Arnoldi et al. 1992, Mari et al. 1995, Sethi et al. 1996). Molecular approaches to parasite detection such as the PCR are rapid, reproducible, and relatively easy to conduct (Andree et al. 1998, Mauel et al. 1996, Sethi et al. 1996). Early and accurate detection of parasites in marine invertebrates is critical, because therapeutic approaches are limited, and avoidance becomes a principal means of disease management and resource protection.

Withering syndrome is a fatal, infectious, bacterial disease of both wild and cultured abalone in California (Friedman et al. 1997). Diagnosis of WS, like many diseases, currently relies on observation of gross signs of affected abalone and detection of the RLP in target tissues by microscopic examination of stained tissue sections. Unfortunately, inclusions of other intracellular bacteria are frequently found in shellfish and are often morphologically indistinguishable from the RLP associated with WS (Friedman and Hedrick, unpubl. obs.). We have confirmed the PCR test differentiates among RLPs in abalone tissues by *in situ* hybridization experiments that utilize the PCR primers as probes (Antonio et al. in press). The probes only hybridize to the RLPs that correlate with the observation of disease symptoms (data not shown). The RLP is, therefore, more easily and rapidly detected by the application of such DNA diagnostic tests as PCR than by microscopy.

This PCR test should prove to be a useful tool for the study of WS, especially for epidemiological investigations of the modes of transmission and reservoirs for the parasite in the marine habitat. In addition, experimental exposure studies combined with this PCR assay should help to identify naturally resistant populations of black abalone. The PCR assay provides direct visualization of specific bands on ethidium bromide stained gels, more rapid diagnosis than microscopic examination of tissue sections, and obviates the need for cell culture isolation of the parasite. Furthermore, we presume the PCR test will detect organisms at lower levels of infection than microscopic examination of stained tissue sections.

The primers designed for PCR detection of the RLP were chosen after alignments showed sequences obtained from *Anaplasma marginales, Ehrlichia bovis, Wolbachia pipientis, Piscirickettsia salmonis, Escherichia coli*, and an unnamed Rickettsia-like bacterium from shrimp found no homologous sites for hybridization. The lack of hydridization of the RLP primers with genomic DNA isolated from bacterial flora cultured from the postesophagus of abalone and from *P. salmonis* genomic DNA from cells grown in tissue culture was indicated by the absence of any amplified products following the PCR. The assay performed equally well on RLP-infected black and red abalone. There was some nonspecific amplification from epipodial tissues of a small number of the black abalone tested but the product (250 bp) was clearly different in molecular weight and sequence from the expected 160 bp amplicon. This 250 bp product may be the result of surface contamination of the epipodium, because it was not observed in red abalone, or black abalone from all locations.

Some heavily infected animals gave positive test results with epipodial tissues; however, in general, most samples of the epipodium were negative by PCR. The relatively weak positives among the epipodium samples may represent cross contamination (with more heavily infected tissues) during sampling or the adherence of RLPs on the epipodium as shed from infected animals in crowded tanks during transport or holding before sample collection. This concern combined with the appearance of the 250 bp amplicon in nonlethal epipodial biopsies discourages us from recommending this sampling approach for detection of the RLP. A more reliable approach is to collect postesophagus or digestive gland tissues from each animal, which requires sacrificing the animal (as do current microscopic procedures).

As mentioned above, an additional application of the PCR primers is their use for *in situ* hybridization (ISH) to visualize the parasite in various tissues or alternate hosts and to differentiate this bacterium from other RLPs commonly observed in marine species (Elston 1986, LeGall et al. 1988). Future work utilizing ISH may also identify portals of entry and early developmental forms not easily seen by standard microscopic examinations, as shown with other parasites of aquatic hosts (Antonio et al. 1999; Antonio et al. in press).

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MICROSTRUCTURE, CHRONOLOGY AND GROWTH OF THE PINTO ABALONE, *HALIOTIS KAMTSCHATKANA*, IN ALASKA

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ABSTRACT The microstructure, deposition of rings, and growth checks in the pinto abalone, *Haliotis kamtschatkana* Jonas, were examined at seven sites in southeast Alaska. Rings were of calcium carbonate with prismatic or block-like structure or were of organic material (called brown rings); sometimes both types were juxtaposed in a compound ring. Rings alternated with nacreous layers having a brick-like or laminar structure. Laminar thickness was correlated with ambient sea temperatures and provided internal evidence of periodicity of ring deposition. One ring a year appears to be deposited in the spire of this abalone in about mid-summer, and a growth check is laid down at the growing edge of the shell in about mid-winter. Rates of deposition of the rings and growth checks were validated by comparison with known growth rates from mark-recapture experiments at one site, and modal analysis of length frequency data at others. Examination of a sample of shells of known age confirmed a deposition rate of one ring a year. Brown rings appear to be laid down adventitiously and were excluded from ring counts for aging purposes. The three independent techniques, rings, growth checks, and modal analysis, gave consistent juvenile growth rates at 7 sites of 14–18 mm/y during the first 4 y. Thereafter, growth rates followed a declining exponential curve. Growth rates differed little between sites, and mean parameters of fitted von Bertalanffy growth parameters for seven sites were: $K = 0.20$; $L_\infty = 125.9$ mm.

KEY WORDS: Chronology, growth rings, growth checks, shell-aging, growth rates, abalone, *Haliotis kamtschatkana*

INTRODUCTION

The molluscan shell is known to cryptically encode, within its microstructure, information on seasonal growth, age, and even habitat relations (Bandel 1990). Knowledge of the microstructure of the abalone shell is accumulating. It is known that the abalone shell deposits prismatic rings in the spire that are useful for aging, and that the shell's aragonitic laminae reflect seasonal temperature changes that confirm the periodicity of ring deposition (Shepherd et al. 1995, Shepherd and Avalos-Borja 1997).

The pinto (or northern) abalone, *Haliotis kamtschatkana*, so named because of its striking color pattern on the epipodium, occurs from southeast Alaska to northern California and was the basis of small commercial fisheries in British Columbia and Alaska until declining stocks caused their respective closures in 1990 (Farlinger and Campbell 1992) and 1995 (Woodby et al. in press). However, recreational and subsistence harvest of the pinto abalone continues in Alaska, and an understanding of the species' population dynamics, growth rate specifically, is necessary for the management of the existing stocks and the rehabilitation of those that have declined.

In this paper, we first describe structural features of the pinto abalone shell. This shell lays down nonpigmented, and occasionally pigmented, rings in the spire, which are clearly visible in horizontal shell sections, and periodic growth checks at the shell's growing edge. We interpret microstructural and ultrastructural properties of the shell to show the periodicity of ring deposition in the shell. We obtained samples of shells from wild populations and estimated the rates of deposition of the rings with size, and the sizes at which checks were laid down. Then we used shells of known age and independently obtained field data on the growth rate to confirm the rate of deposition of the rings and checks. We show that rings are laid down in summer and checks in winter and apply the techniques to estimate the growth rate of this abalone at a number of sites in southeast Alaska.

MATERIALS AND METHODS

Data Collection

Shell samples of the pinto abalone were collected by diving from seven sites in southeast Alaska (Fig. 1). At each site, divers searched intensively from the shallow sublittoral to the lower depth limit of the abalone at a depth of 10–15 m and specifically under boulders for all abalone in the size range 0–100 mm shell length (SL), and at some sites above 100 mm as well. We also collected the dead shells of abalone encountered.

Using the method of Shepherd et al. (1995), we took horizontal sections of each shell by grinding the spire with a disk grinder until a minute hole appeared in the shell, then polishing the section with sequentially finer abrasive (500–1,200 grit), and finally etching the surface with dilute HCl. The horizontal section reveals a series of concentric prismatic layers (called rings), laid down alternately with aragonitic nacre. The number of rings in each section was counted under a low-power binocular microscope. The prismatic layers are opaque and sometimes faintly honey-colored. Examination of the section showed clearly whether the most recently laid material was nacreous or prismatic. A few shells (2% of the total), bored around the spire by boring bivalves or annelids, were unreadable and were discarded.

Vertical sections of a subsample also were cut across the midpoint on the spire with a slow-speed electric saw with a diamond disc, were polished and were etched as described above, and then were cleaned in an ultrasonic bath for further analysis.

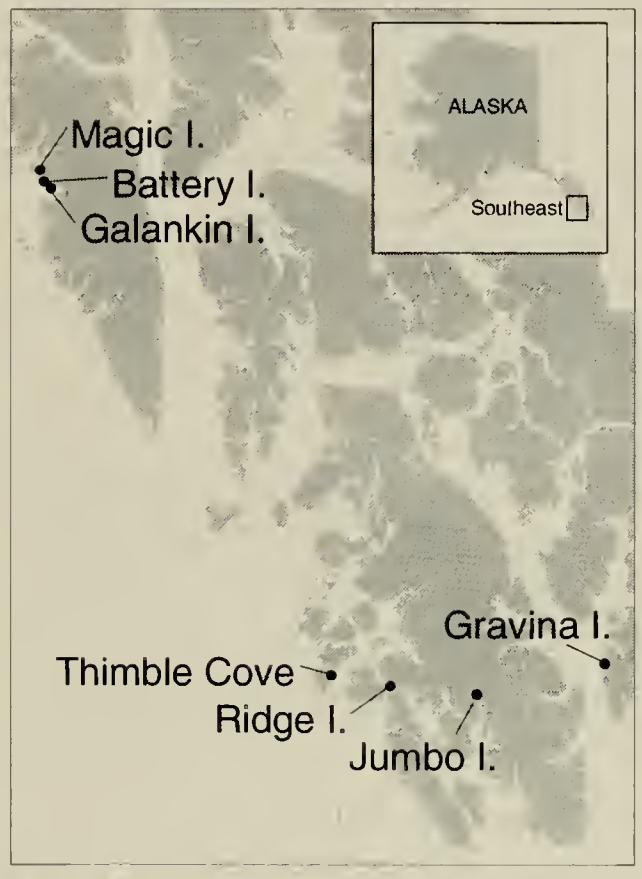

Figure. 1. Map of southeast Alaska showing sampling sites. Locations: Magic Island, 57°05′45″/135°24′07″; Battery Island, 57°03′26″/135°22′55″; Galankin Island, 57°02′04″/135°20′05″; Thimble Cove, 55°18′46″/133°34′42″; Ridge Island, 55°16′26″/133°12′18″; Jumbo Island, 55°14′37″/132°39′55″; and Gravina Island, 55°21′13″/131°51′23″.

To examine the variation in crystalline ultrastructure across the inner nacreous layer of vertical sections, we made a transect from the outer to inner shell surface at right angles to the prismatic layers and a series of micrographs (×5,000) were taken under a scanning electron microscope (SEM) (JSM5300, JEOL). The thickness of aragonitic laminae was measured at two sites on each micrograph with three replicate measurements per site. Each measurement was of 10 adjacent laminae from which a mean laminar width was calculated for each site. We chose a transect location on the vertical section where the rings were evenly spaced and took 3–5 micrographs at about equal intervals between each ring. The main elemental composition of rings was examined by X-ray microanalysis with energy-dispersive spectroscopy. In all, nine vertical sections were examined in detail with SEM.

We compared seasonal variation in sea surface temperatures with laminar widths to elucidate the periodicity of ring deposition. Monthly mean temperatures were derived from optimally interpolated weekly surface observations and satellite measurements at 1° resolution (Reynolds and Smith 1994). The data set was provided by the National Center for Atmospheric Research and was developed at the National Center for Environmental Prediction of the National Oceanic and Atmospheric Administration.

Pinto abalone, presumptively 0–4 y old, lay down growth checks (described in Results), which are best seen with transmitted light (Fig. 7) in shells to about 70–80 mm SL. Larger shells appear also to lay down growth checks in the shell, which may be seen by incident light; however, they are not clear, and their interpretation was difficult and outside the scope of this study.

We measured SL to the 1st, 2nd, 3rd, and 4th growth checks (SL_1, SL_2, SL_3, and SL_4, respectively) where these checks were visible for shells from four sites with sufficient data. Occasionally, a double check or another secondary, less conspicuous check was observed between these checks. In the former case, length to the checks was averaged, and in the latter case where we were uncertain as to which was the primary check, we measured the SL to the former of the two. In order to estimate when the growth checks were laid down, we first estimated for each site (except for Battery Island and Jumbo Island, for which there were insufficient data) the mean length at a presumed age of 1 y. These lengths were extracted from Table 1 for three sites and from the mean length of shells with one ring at Galankin Island. We then estimated the mean proportion of the annual growth achieved before deposition of the first growth check. By assuming that the growth checks were laid down exactly 1 y apart, and that the site-specific growth rates were those calculated in Table 2, we calculated the proportion of the 2nd, 3rd, and 4th year's growth achieved before deposition of the respective growth check for that year.

We examined seven shells of known age. These abalone were taken from the wild at Sitka at a size of about 50 mm SL in the summer of 1978 and were maintained in aquaria at Seward. The shells were estimated from growth checks to have been about 3 y old at capture and were assigned a birth date of July 1, 1975. The abalone died between 1985 and 1994, and the year of death was recorded on the shell. We assumed that each abalone died on July 1 of the year marked on the shell. In addition, there were two shells marked at 82 and 92 mm SL, respectively, and at liberty off Dempster Island in British Columbia for 5 y. We estimated the number of rings present at the date of tagging, from length, in the light of the known growth rate for that site, and by deduction from the number present at the date of capture, and the estimated number of rings deposited during the period between dates of tagging and recapture. Growth checks in the shells were counted, and the presence of checks at lengths near those recorded for SL_1–SL_4 in Table 2 was inferred where not visible.

Statistics

We estimated the growth rate by two methods, both independent of interpretation of ring counts and growth checks. First, where there were adequate length frequency data, we used the EMMIX program to separate modes. The procedure fits Gaussian curves and uses maximum likelihood methods to separate them (MacLachlan and Peel 1998, MacLachlan et al. 1999). The modes were assumed to be annual year classes because the pinto abalone has a narrow summer spawning season (reviewed by Sloan and Breen 1988). Modes are conspicuous for at least the first 4 y, but less so after that. We followed Fournier and Breen (1983) and Breen and Fournier (1984) and considered that the first mode at 15–25 mm SL appearing in summer samples was the 1-y-old cohort and that each succeeding mode was 1 y older. We estimated growth rates from modal intervals at several sites. Second, we examined a subset of mark-recapture data from Gravina Island where the period between dates of tagging and recapture was about a year (Woodby et al. in press) and derived mean juvenile growth rates for that site.

In the regressions of length versus number of rings, there is an unknown measurement error in the count of rings, suggesting that

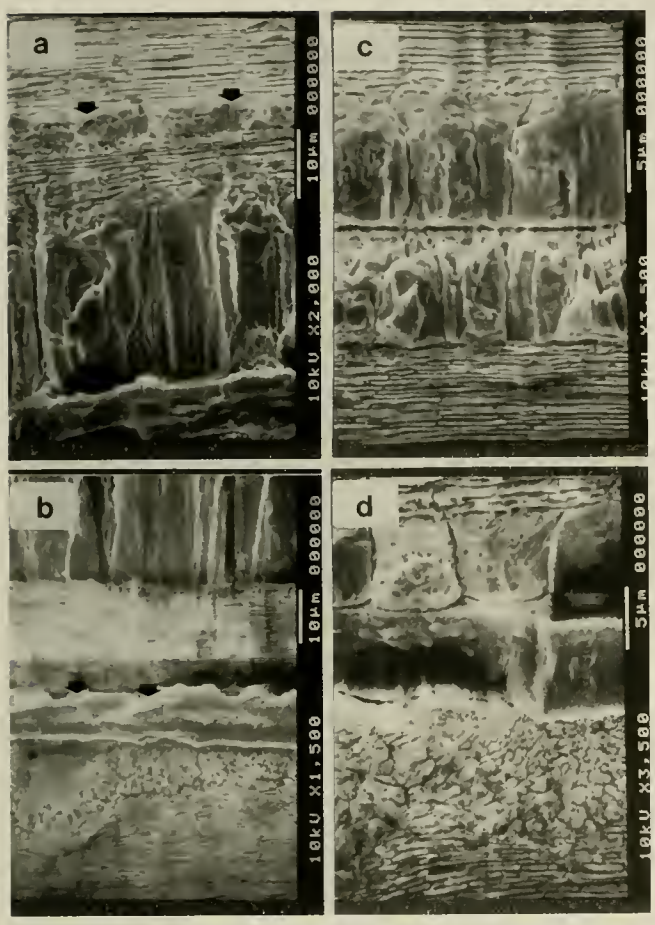

Figure. 2. Types of rings in *H. kamtschatkana*. The deposition of shell, and the descriptions below, go from top to bottom in each micrograph. a) Successive layers of nacreous laminae, a minor ring (arrows), more laminae, and the first major ring of block-like prismatic structure. b) Successively, a major ring, nacreous laminae, and a brown ring of organic material (arrow) followed by irregular material and lastly nacreous laminae. c) Nacreous laminae, followed by a double ring, and then more laminae. d) A compound ring comprising block-like prisms followed sequentially by an organic brown ring, irregular material and, last, nacreous laminae.

the regression should be formulated as an "error-in-variables" model (Model II, see Ricker 1973). However, the regressor variable is not normally distributed, hence the methods of solution are complex (Fuller 1987). Acknowledging this problem, and assuming that the measurement error is small, we note that the slopes (growth rates) at each ring interval may be slightly biased toward zero when formulated as a standard linear regression.

On the assumption that rings were laid down annually, von Bertalanffy growth parameters and standard errors were estimated with a Gauss-Newton nonlinear regression (SAS 1996).

RESULTS

Microstructure

The rings of *H. kamtschatkana* show variation in microstructure. The initial minor ring seen in a proportion of shells under optical microscopy was usually separate from but occasionally juxtaposed to the first major ring overall or to part of its length in the section (Fig. 2a), so that it may not be distinguishable from the major ring at low magnification. SEM microscopy revealed that

TABLE 1.

Regression equations of SL versus presumptive age in years (A) for data on modal means of length-frequency distributions and presumptive age.[a]

Site	Regression Equation (SE)	R^2	Length at Age 1 y (mm)
Gravina Island	SL = 17.0 + 11.0 (0.8) A	0.99	[b]
Magic Island	SL = −3.4 + 18.5 (1.3) A	0.99	16.3
Thimble Cove	SL = 7.9 + 13.8 (0.2) A	1.00	21.5
Ridge Island	SL = 12.2 + 13.1 (0.8) A	1.00	22.3

[a] The age at one year is the modal mean of the initial mode in the length-frequency distributions.
[b] No 1-y-old animals were found.

rings were of two kinds. Most were of calcium carbonate, were unpigmented, were of simple, prismatic, or block-like structure with very little organic material (Fig. 2a, c), and were presumed to be composed of aragonite and/or calcite, as is found in other abalone species (Dauphin et al. 1989, Hawkes et al. 1996). The second type of ring contained relatively little calcium but was high in carbon, sulphur, oxygen, silicon, and sodium, indicating a likely organic composition (Fig. 2b, d); these rings were pigmented when viewed under the optical microscope and are termed brown rings. The brown rings were sometimes isolated from other rings and sometimes juxtaposed on one side or the other of a prismatic ring to form a compound ring (Fig. 2d).

The width of minor rings was 4–9 µm, and they were readily distinguishable by size from major rings, which were 10–46 µm across. Brown rings were 10–15 µm across and were visible even under low-power optical microscopy by their honey color. They could be confused easily with compound rings.

Mean laminar thickness measured along transects running transverse to the rings varied in a cyclic manner between rings. A plot of the change in the thickness of laminae along a transect (Fig. 3a) shows a decline in thickness soon after deposition of the ring and a later increase prior to deposition of the next ring. This pattern was repeated between rings and was the typical pattern in the sections examined. The thickness of laminae was significantly correlated with sea temperature, assuming that the rings were laid down in mid-summer (see below). The highest correlations occurred when a lag period of 1 mo ($r = 0.59$; $P < 0.001$) or 2 mo ($r = 0.69$; $P < 0.001$) were applied to sea temperature data.

We also examined a parasitized shell in which a brown ring and two compound rings were present. Assuming the same relationship with temperature, we estimated the timing of ring deposition from the laminar thickness. The results (Fig. 3b) show that the brown ring interrupted the pattern of seasonally changing thickness of laminae, which is consistent with our conclusion that they are adventitious. In this shell, the highest correlation between temperature and laminar thickness occurred with a lag period of 2 mo for sea temperature ($r = 0.67$; $P < 0.001$) compared with $r = 0.64$ ($P < 0.001$) for a 1-mo lag period and $r = 0.43$ ($P < 0.05$) with no lag.

Ring Deposition

At low magnification, horizontal and vertical sections of the shell show distinct growth rings. Shells of 13–28 mm SL were considered to be 1-y-olds (see below), and a proportion of them showed a fine outer ring (termed minor ring) as well as the sub-

TABLE 2.

Lengths to growth checks SL_1, SL_2, SL_3, and SL_4 for shells of *H. kamtschatkana* at six sites.

Site	*N*	SL_1 (SE)	*N*	SL_2 (SE)	*N*	SL_3 (SE)	*N*	SL_4 (SE)	Mean Growth Rate SE (mm/y)
Gravina Island	26	12.0 (0.5)	39	27.5 (0.4)	40	42.7 (0.6)	28	57.5 (0.9)	15.1 (0.2)
Magic Island	27	11.4 (0.6)	29	26.1 (0.8)	23	44.6 (0.6)	18	61.4 (0.6)	16.9 (0.5)
Thimble Cove	42	11.5 (0.3)	42	26.2 (0.5)	28	41.6 (0.6)	12	56.3 (1.1)	15.0 (0.1)
Galankin Island	13	11.8 (0.5)	11	27.6 (0.9)	13	44.1 (0.9)	13	57.8 (0.9)	15.5 (0.4)
Ridge Island	22	10.9 (0.4)	46	26.6 (0.5)	52	42.5 (0.5)	31	55.3 (0.8)	14.9 (0.5)
Battery Island	3	14.0 (1.7)	6	25.5 (1.1)	8	47.1 (1.1)	8	61.4 (1.0)	16.4 (1.3)
Jumbo Island	7	11.8 (0.6)	16	25.3 (0.6)	21	40.6 (0.8)	16	53.6 (0.8)	14.1 (0.3)
Mean values		11.9 (0.4)		26.4 (0.3)		43.3 (0.8)		57.6 (1.1)	15.4 (0.4)

Individual lengths are not independent within a site because individual shells have multiple growth checks.

sequent thicker rings (termed major or prismatic rings). In *H. kamtschatkana*, the spire is unusually elevated some 2–5 mm above the surrounding shell, compared with Australian or Mexican species (unpublished observations) and, hence, more susceptible to erosion than those species. The incidence of minor rings did not decline in shells to a presumed age of about 4 y but thereafter declined rapidly, and none were seen in shells > 5 y old. We presume that the decline in their incidence was due to erosion, because with increasing age the minor ring disappeared from the face of the horizontal section but could still be seen on the eroded lateral margin of the spire.

The incidence of separate minor rings also varied between sites. At Galankin Island, 62% of shells had minor rings, and at Magic Island, 48%, but at Thimble Cove, 17%, at Ridge Island, 13%, and at Gravina Island only 2% had minor rings. It is possible, of course, that minor rings may have occurred juxtaposed to their neighboring major rings (Fig. 2a), in which case we would not have distinguished them. As some presumptive 1-y-old shells had both a distinct minor and a major ring, we concluded that both were laid down in the first year, the minor ring possibly during the first winter when the first growth check was deposited (see below). We examined a sample of presumptive 1-y-old shells from the two sites with the largest proportions of shells with minor rings to see if deposition of a distinct minor ring was related to length. The mean length of shells that deposited a distinct minor ring was 21.0 mm (SE 1.6 mm), and of those that did not, was 15.2 mm (SE 1.1 mm). The differences were significant ($t = 2.4$; $P < 0.05$). This suggests that individuals, which either grew faster or settled earlier in the summer (or both), were more likely to lay down a distinct minor ring than those that grew more slowly or settled later. As the presence of minor rings was variable, and they were in any event superfluous for aging, we excluded them from further consideration.

Examination of the shell nacre on the ventral surface at the spire indicated whether nacre or a ring (visible as an opaque layer with a nonreflective surface) was the last layer deposited there. In all, at four sites 91.4% of shells ($N = 358$) had most recently deposited a ring over a broad area near the spire. In the remaining cases, some nacre had been more recently laid down at the spire. We concluded that a ring must have been laid down during the last episode of shell deposition before the collection of the samples in mid to late July at all our sites.

A practical problem that we experienced in estimating the age of juvenile shells from ring counts, mainly at the Magic Island and Thimble Cove sites, was the difficulty in distinguishing minor from major rings. Thus, it was possible to interpret a shell with two rings as a 1-y-old with a minor ring or a 2-y-old without one. The presence of two growth checks (see below) in 2-y-old shells helped to resolve this dilemma. Another problem was ambiguity in ring counts. This happened where two rings merged or where there were false rings, i.e., where one ring divided into two and then merged again into one (see Fig. 1d in Shepherd et al. 1995). In these cases of uncertainty as to whether there was one or two rings (6% of all shells), the ring count was revised in the light of the number of growth checks observed. In another 2% of all shells examined, there was a clear discrepancy of 1 year in age estimated from ring counts and from growth checks. In these cases, we adopted the ring count for the purpose of the regressions, although they were not consistent with the counts of growth checks. In very few cases (1.4% of all shells), the exposed section was milky, and no rings were visible.

The growth rate of many species of abalone is linear or nearly so for the first 3–5 y of life, and thereafter is curvilinear. The linear phase can be fitted with a linear regression, and the curvilinear phase with a von Bertalanffy curve (Shepherd and Hearn 1983). Hence, a regression of SL versus the number of rings during the linear phase of growth will give an estimate of the rate of deposition of rings with age if the growth rate is known (Shepherd and Triantafillos 1997).

Plots of length versus number of rings showed a linear or nearly linear relation with length to about 80 mm SL at each site after which the curve approaches an asymptote as expected for von Bertalanffy growth. Linear regressions were fitted to data for each site for the linear phase of growth. The regression equations are given in Table 3 and are plotted in Figure 4 for those sites where we found the most juveniles. The deposition rate of rings with length was similar at all sites. Assuming that the deposition rate of rings is age-related, then the growth rate appears to be fastest at Magic Island and Jumbo Island and slowest at Gravina Island.

Mark-recapture data from Gravina Island over the initial length range of 45–75 mm SL (Fig. 5) show that the growth rate declined linearly from about 15 mm/y at 50 mm SL to about 5 mm/y at 75 mm SL (Woodby et al. in press). The mean growth rate of marked individuals over this length range was 10.3 mm/y (SE 0.9 mm/y). This is close to the mean growth rate of 11.1 mm/y (SE 0.7 mm/y) given in Table 3 for Gravina Island. Although the two estimates cannot be compared statistically, their SEs overlap, suggesting that they are not significantly different. Tagged abalone at Gravina Island showed von Bertalanffy rather than linear growth (Fig. 5), which is inconsistent with our hypothesis of linear juvenile growth rate and with our analysis of length-frequency data. This incon-

TABLE 3.

The seven sampling sites, with regression equations of SL versus number of major rings (R) of *H. kamtschatkana* for all shells <80 mm SL.[a]

Site	*N*	Regression Equation (SE)	R^2	Length at Age 1 y (mm)	Brown Rings (%)
Gravina Island	56	SL = 19.8 (2.7) + 11.1 (0.7) R	0.80	29.9	27
Magic Island	45	SL = 2.5 (2.5) + 16.7 (0.8) R	0.90	19.1	13
Thimble Cove	86	SL = 8.2 (1.6) + 13.7 (0.5) R	0.90	21.8	14
Galankin Island	32	SL = 8.3 (2.9) + 13.8 (0.8) R	0.90	22.1	14
Ridge Island	70	SL = 10.0 (2.4) + 13.3 (0.7) R	0.84	23.3	33
Battery Island	9	SL = 3.2 (7.2) + 18.6 (2.4) R	0.90	21.8	17
Jumbo Island	28	SL = 24.9 (5.5) + 10.1 (1.4) R	0.67	35.0	19

[a] Estimated mean growth rates and length at a presumed age of 1 y were derived fom the regression equations. The mean incidence of brown rings in shells >70 mm SL are given for each site. The slope of the regression equation is the mean annual growth rate (mm per year).

sistency could be an artifact of insufficient recaptures of tagged abalone that were tagged at < 50 mm SL.

Length-frequency distributions for four sites are shown in Figure 6 with Gaussian curves fitted to the prominent modes. Linear regressions of modal means versus presumptive age gave estimates of the mean growth rates (Table 1). These were 13–19 mm/y, and length at 1 y of age was 16–23 mm, according to the site. High R^2 values of > 0.99 for every site indicate that the growth rate was uniform and very nearly linear. The two sets of estimates of growth rates for the four sites, summarized in Tables 1 and 3, were very close with overlapping SEs, so we accepted that one ring per year was laid down at these sites. Mean lengths at one ring (Table 3) and at 1 y (Table 1) were also very close (we found no 1-y-olds at Gravina Island), which is consistent with the hypothesis that one ring per year is deposited.

Growth curves for all length-age couplets for each site and for all sites combined are given in Table 4. Length-age couplets and a growth curve for all sites combined are plotted in Figure 9.

Brown Rings and Shell Erosion

As shown by SEM micrographs and energy-dispersive spectroscopy, brown rings are qualitatively different in structure and composition from prismatic rings. An examination of a series of vertical sections showed that brown rings were laid down only in the presence of infestation by endobionts and that the number of brown rings increased with increasing infestation. Further, they appeared to have been laid down independently of the usual regular pattern of deposition of prismatic rings, and so they were considered adventitious when alone rather than substitutional for a prismatic ring. Accordingly, we excluded brown rings from the count of rings for the purpose of aging shells when they occurred alone but not when they occurred as compound rings, i.e., when they were juxtaposed to a prismatic ring. Shepherd and Huchette (1997) similarly found that brown rings in *Haliotis scalaris* were adventitious. In horizontal sections, brown rings were usually rec-

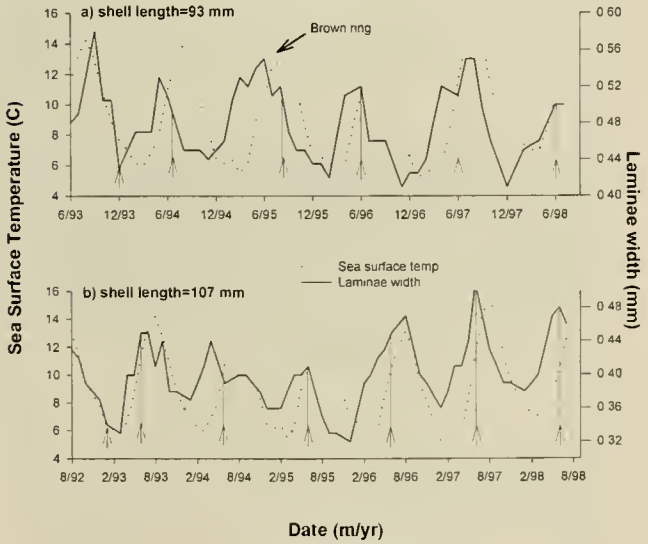

Figure 3. Change in laminar width in vertical sections in the spire of *H. kamtschatkana* for: a) a 5-y-old shell (93 mm SL) that had a brown ring between the first and second major rings, and b) a 6-y-old shell (107 mm SL). Arrows indicate the location of rings in the sequences, and the first arrow in each sequence is a minor ring.

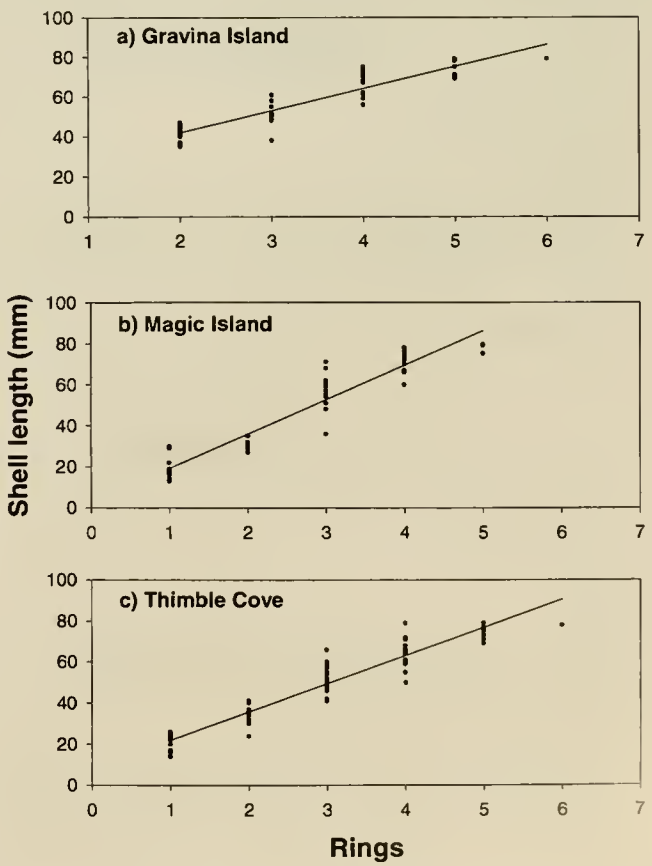

Figure. 4. Plots of length (mm) versus the number of rings of *H. kamtschatkana* at Gravina Island, Magic Island, and Thimble Cove.

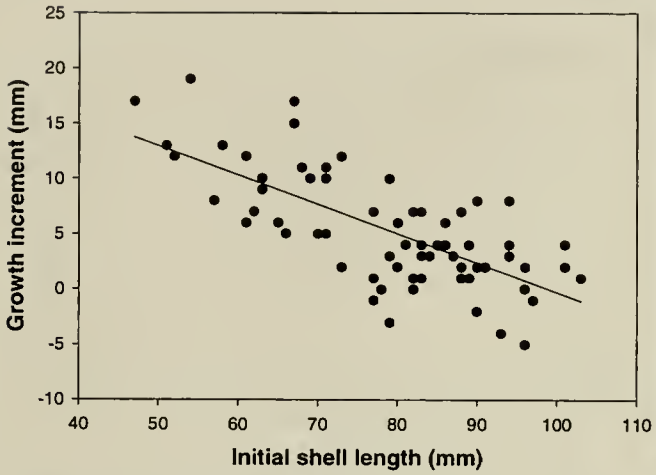

Figure. 5. Plot of annual increment (mm per year) versus the initial length of marked *H. kamtschatkana* at Gravina Island.

ognizable because the process of grinding and polishing caused cavities on the surface where the softer organic material was preferentially excavated.

Brown rings were rarely present in shells < 70 mm SL. Above

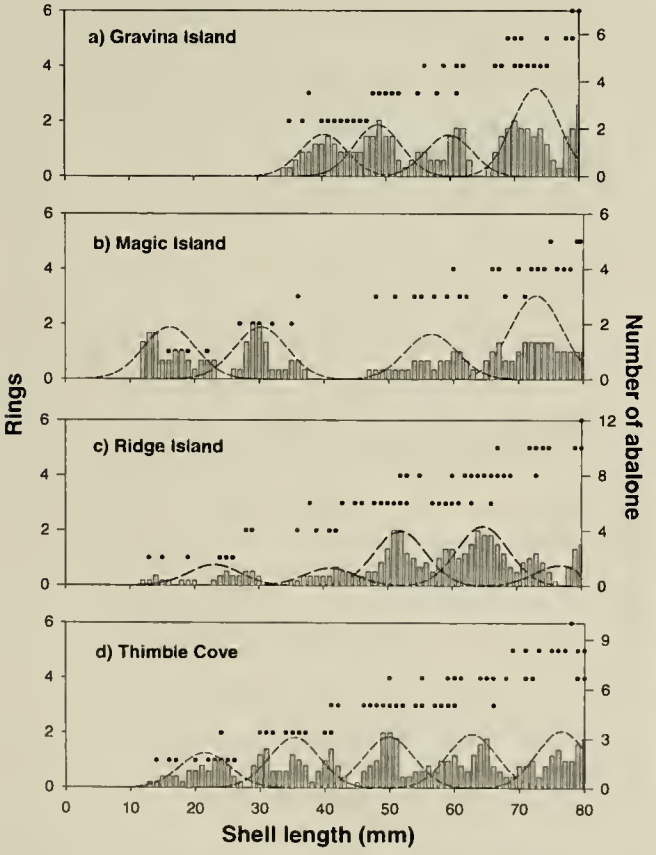

Figure. 6. Plots of length-frequency data (vertical bars are running means of 3-mm size intervals), rings at length (solid circles) and Gaussian modes fitted by EMMIX (dashed lines). Modal means are: a) Gravina Island: 40.3, 48.7, 59.8, and 73.3 mm, variance 13.0 mm; (b) Magic Island: 16.3, 30.5,56.6, and 72.7 mm, variance 16.1; c) Ridge Island: 23.1,41.0, 52.0, 64.8, and 76.7 mm, variance 15.8; and d) Thimble Cove: 21.5, 35.3, 49.9, 62.7, and 76.5 mm, variance 14.9.

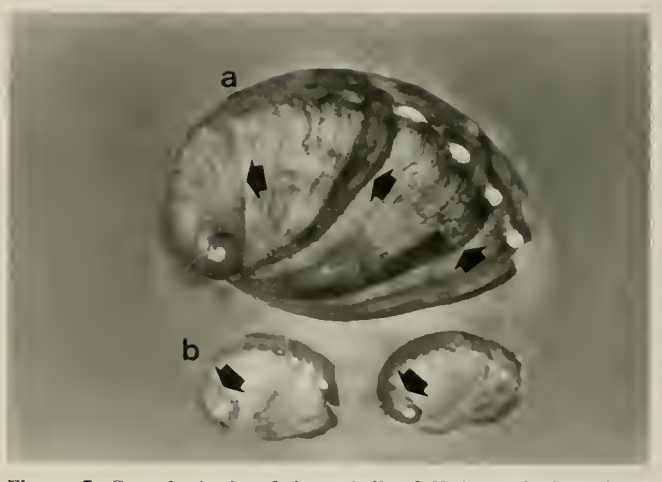

Figure. 7. Growth checks of three shells of *H. kamtschatkana* from Thimble Cove seen by transmitted light. A) SL = 66 mm. Three checks indicated by arrows. a) SL_1 not visible; SL_2 = 28 mm; SL_3 = 42 and 46 mm (a typical double check); and SL_4 = 60 mm. B) SL = 25 mm (left); and SL = 26 mm (right). For both shells $SL_1 \approx 10$ mm (indicated by arrows) and $SL_2 \approx 25$ mm (faintly visible at the edge of the shell).

that size, the incidence of brown rings in the spire sections of shells in samples varied from 13–33% according to site (Table 3). Overall, however, only 3% of shells > 70 mm SL were unreadable because of the effect of parasites alone or were compounded by erosion of the shell.

Shell erosion was slight (2–5%) at our study sites, which were all in places sheltered from ocean swell. Generally, erosion of the shell was associated with attacks by parasitic endobionts, which were provisionally identified as polydorid annelids, and occasionally the bivalve, *Penitella* sp., which caused excavations around the elevated spire. In most cases, the eroded rings could still be seen and counted, not in the horizontal section, but at the eroded margin of the spire near the prominent suture line that joins the older shell with the later deposited shell.

Growth Checks

Growth checks, which are lines marking an interruption in shell growth, were characterized by at least one, but usually more, of the

Figure. 8. Lateral view of two shells of *H. kamtschatkana* showing a suture line in the shell that marks a growth check continuing below the line of pore-holes to the margin of the shell. a) SL = 50 mm (SL_2 indicated by arrow). b) SL = 41 mm (SL_2 indicated by arrow).

following features as seen by transmitted light (Fig. 7): a ridge up to 0.5 mm high dorsally on the shell; and a fine suture line in the shell or a discontinuity in the pattern of pigmentation or sculpture of the shell. The growth checks were apparently formed at the growing margin of the shell at the time of interruption to shell growth. The growth check continued past the pore-holes to the margin of the shell where the discontinuity or ridge was often more conspicuous (unless eroded) than elsewhere (Fig. 8). The first check, SL_1, is lost almost always by age 3 y, and the next check, SL_2, is lost a year later as new nacre is deposited on the ventral surface of the shell, preventing transmission of light through the shell. Checks SL_3 and SL_4 are often visible dorsally only as discontinuities in the shell.

The estimation of age from growth checks alone has some limitations or ambiguities. In 8% of the shells that are > 5 y old, one or more checks were missing, apart from the incremental loss of the earliest ones. In 6% of the shells over the same age range, dual growth checks, i.e., two checks very close together, were seen, most often at Sl_2 and SL_3. In these cases, we measured length to the check that persisted to the shell margin or to the former of the two checks, if they were indistinguishable. In about 1% of the shells, no checks could be seen at all.

The mean lengths at which the growth checks SL_1–SL_4 were laid down are given in Table 2. These were compared to estimates of the lengths at age 1 y from the regressions in Table 1 for the four sites listed and from the mean length of shells with one ring for Galankin Island. For these five sites, a mean value of 60% of the first year's growth was achieved before the growth check was laid down, declining to a mean of 41% in the fourth year. The decline was not significant ($t = 0.20$). Overall, for the five sites 53% of the annual growth during the first 4 y was achieved before deposition of the growth check for the respective year. Assuming a uniform birth date in July, we conclude that the growth check was laid down about half way through the year, i.e., in about January, which is about mid-winter with minimal sea temperatures.

The mean lengths at which growth checks were laid down (Table 2) are very nearly linear with age ($r^2 > 0.99$ for all five sites), indicating a linear growth over the length range of ~10–60 mm SL. The growth rates derived from growth check analysis (Table 2) are highly correlated with those calculated from ring analysis (Table 3) ($r = 0.88$; $P < 0.01$), and the mean growth rates for all sites combined derived from the two independent methods did not differ significantly ($t = 1.24$). The consistency in growth rates between the ring and growth check analyses supports the hypothesis that growth checks are deposited annually.

TABLE 4.

Parameters of von Bertalanffy growth curves fitted to length-age data for each site.

Site	*N*	K (SE)	L_∞ (SE)
Gravina Island	86	0.21 (0.02)	119.7 (5.6)
Magic Island	103	0.20 (0.01)	129.6 (4.4)
Thimble Cove	110	0.16 (0.01)	136.9 (7.7)
Galankin Island	41	0.22 (0.03)	113.0 (7.7)
Ridge Island	79	0.16 (0.02)	134.9 (13.7)
Battery Island	32	0.25 (0.02)	118.9 (4.2)
Jumbo Island	58	0.19 (0.02)	128.6 (5.5)
Mean value		0.20 (0.01)	125.9 (3.4)
All sites combined	509	0.18 (0.01)	131.9 (2.4)

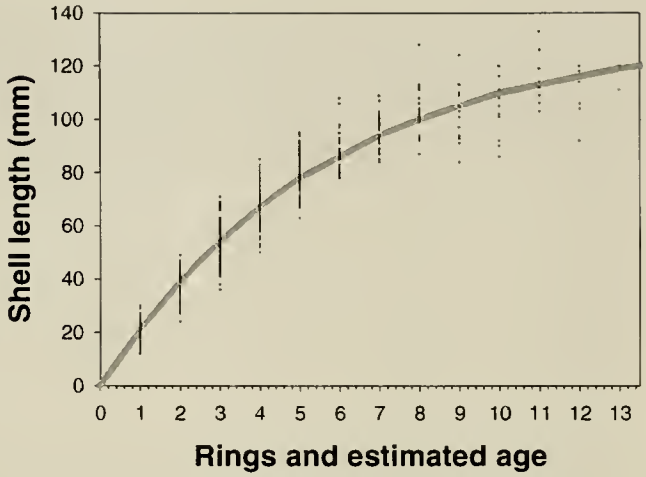

Figure. 9. A plot of a von Bertalanffy growth curve fitted to length-at-age data for all sites combined, and length-increment data from Gravina Island.

The number of rings and growth checks present in shells of known or estimated age are given in Table 5. Of the seven shells of known age, three were eroded and/or parasitized at the spire and the rings could not be counted. The remaining four deposited a mean number of 1.03 rings per year (SE 0.02 rings per year), after deducting the number of brown rings, over a mean period of 14.5 y/shell. The seven shells also laid down an estimated 0.92 growth checks per year. The two shells from British Columbia each laid down an estimated five rings during the 5 y between tagging and recapture, but growth checks were not visible in these shells.

DISCUSSION

Microstructure

The microstructure and pattern of changes in thickness of aragonitic laminae show striking similarity to those of *Haliotis corrugata* and *Haliotis fulgens* Phillipi. The block-like prisms and the brown rings (described by Shepherd and Avalos-Borja 1997 as being of granular structure) are similar in the three species, although in *H. corrugata* the rings were compound, with prismatic rings and brown rings regularly juxtaposed. The similarity between the three species is perhaps not surprising, given their recent common ancestry (Brown 1993, Lee and Vacquier 1995).

The cyclic change in the thickness of the laminae between rings and the correlations with temperature provide internal corroborative evidence that laminar thickness is temperature-dependent and, hence, that ring deposition is annual. The significance (if any) of the 1–2-mo lag correlation is unclear. We arbitrarily assumed that laminar deposition occurred continuously throughout the year. If, however, there was a pause in laminar deposition for 1–2 mo after sea temperature reached a maximum, as could happen around the time of spawning, then an apparent lag would be observed. This question cannot be resolved until laminar deposition can be accurately pinpointed in time as with a time stamp (see Hawkes et al. 1996). The dependence of laminar thickness on temperature has been previously noted for the abalone *H. corrugata* (Shepherd and Avalos-Borja 1997) and *H. fulgens* (Shepherd et al. 1995), as well as some bivalves (reviewed by Lutz and Rhoads 1980). The effect is analogous to the control of width of tree rings by air temperatures (Briffa et al. 1995) and to the differential density banding in

TABLE 5.

Number of rings and growth checks in shells of *H. kamtschatkana* of known or estimated age.[a]

Shell Length (mm)	Age (y)	No. of Rings	No. of Checks (Inferred + Counted)	Comments
103	10	10	4 + 8	Two extra brown rings excluded
103	12	12	1 + 12	Two extra brown rings excluded
106	15	8?	2 + 12	Spire partly destroyed by erosion and parasites
98	17	18	3 + 10	Two extra brown rings excluded. Eroded in part
96	18	13?	2 + 11	One extra brown ring excluded. Spire partly destroyed
98	19		2 + 17	Spire completely destroyed
102	19	20	2 + 15	Two extra brown rings excluded
99[b]	10.2 ± 0.3	10		Shell from Dempster Island, B.C.
114[b]	10.8 ± 0.3	11		Shell from Dempster Island, B.C., remnants of two rings seen at eroded margin of spire near suture line

[a] The first seven shells were held in aquaria at Seward, Alaska until they died. The presence of growth checks (where not visible in the expected position to age 3.5 years) was inferred; i.e. 4 + 8 means 4 rings were inferred and 8 later ones counted.
[b] Both tagged abalone at liberty for 5 y. Age at tagging was estimated from a mean growth rate of 15.9 mm/y derived from the regression of the number of rings versus SL for a sample of shells ($N = 21$) from the tagging site, assuming that one ring per year was laid down.

corals due to seasonal sea-temperature changes (Dodge and Lang 1983).

Shell Aging

A number of studies have shown that rings can be reliably used to age the shell of various species of abalone (Prince et al. 1988, Erasmus et al. 1994, Shepherd et al. 1995a, Shepherd et al. 1995b, Shepherd and Avalos-Borja 1997, Shepherd and Huchette 1997, Shepherd and Triantafillos 1997, Shepherd and Turrubiates 1997). On the other hand, only four species are known to lay down growth checks that are useful for aging (reviewed by Day and Fleming 1992, Shepherd et al. 1995b). Previously Shepherd and co-authors (cited above) have considered that ring counts may not give a precise age for each individual shell due to apparent random variability in the deposition rates between individuals, although they do give a valid estimate for a population. The low incidence of clear inconsistency (~2%) in estimates of age between the two methods increases confidence in each and suggests that the rate of deposition of rings is relatively precise for individuals of this species, although slight ambiguity in interpreting structures exists with both methods. Thus, ring deposition satisfies the criteria proposed by Day and Fleming (1992) that deposition rates must be consistent and that deposition is at about the same time each year. Similarly, in species that lay down growth checks, a small percentage of shells fail to do so in any year. The use of both methods in the pinto abalone has the advantage that each method provides an independent test of the accuracy of the other, since both rings and growth checks are laid down according to different external cues.

On the question of whether horizontal or vertical sections at the shell spire give better readings, we note that, although the two kinds of section show spatial effects in different planes, the readings from each are comparable (see Shepherd et al. 1995). We prefer horizontal sections for the practical advantages of ease of preparation and reading (especially fine rings). An advantage of vertical sections is that brown and compound rings are more easily distinguishable under higher magnification.

Rings appear to be laid down during maximum summer temperatures or spawning (or both) as Shepherd and co-workers have found for Mexican and Australian species. Conversely, growth checks are apparently deposited during minimum temperatures, as is known for other cold-temperate species (Forster 1967, Sakai 1960, Poore 1972) and one curious subtropical species (Shepherd et al. 1995b).

The exclusion of brown rings from counts for aging purposes is rarely problematic. Brown rings can most readily be distinguished in horizontal sections by the cavities that commonly surround them (caused by the rasping process, which preferentially excavates softer organic matter) and by their rupture of the pattern of deposition of rings. Color alone is ambiguous because of the frequent occurrence of brown rings in juxtaposition to prismatic rings at least over some part of the length of the latter. Usually, the prismatic and organic elements of a compound ring can be seen at about ×20 magnification or more.

We do not exclude the possibility of using growth checks to estimate the age of shells > ~4 y, but we simply point out that ambiguity in identifying checks seems to increase with age and may nullify the benefit of an independent aging method. We note that a few shells of known age had fewer than the predicted number of growth checks (Table 5), suggesting the possibility of some bias. We also observed that growth checks were less conspicuous in shells from British Columbia than in those from Alaska. Possibly higher sea-temperature minima in British Columbia cause a briefer recession or no cessation of winter growth. Another source of bias in aging abalone is through the (usually) combined effect of parasites, which cause deposition of brown rings, and shell erosion. This was not problematic in this study because few pinto shells had brown rings in the length range of interest.

Growth Rates

Our study provides the most detailed information on the growth of the pinto abalone in Alaskan waters. The growth rate of this abalone is poorly known in the first 4 y of life but is better known for older individuals in British Columbia waters (reviewed by Sloan and Breen 1988). Paul et al. (1977, Fig. 4) estimated a growth rate of ~17 mm/y in the laboratory for individuals maintained at 12–15°C, a rate later confirmed in the laboratory by Sloan and Breen (1988). The latter authors presented an equation relating the growth rate of 30 mm SL abalone with water temperature. The mean annual sea surface temperature range at Sitka is ~4.5–14°C (Standley 1987), and the monthly mean is 8.2°C. This would be somewhat higher than the mean temperature at 5–10 m depth

where this abalone lives. Application of their formula gives a mean growth rate of 18.0 mm/y, which is slightly greater than those found in this study. A study by Larson and Blankenbeckler (1980), which is cited in Sloan and Breen (1988), found that the growth rate decreased with increasing size. Annual length increments averaged 19.1 mm for individuals having < 50 mm SL, 12.6 mm for those of 50–74 mm SL, 6.2 mm for those of 75–99 mm SL, and 4.3 for those of > 100 mm SL. Quayle (1971) estimated that the pinto abalone had a 35-mm SL at age 2 y and thereafter grew at a rate of 10 mm/y. Fournier and Breen (1983) estimated that growth rates from 1–5 y ranged from 11–16 mm/y at two sites; these estimates were based on the decomposition of "snapshot" length-frequency data into annual modes. Our data are more accurate than these estimates but are within the same range.

Like Fournier and Breen (1983), we do not have direct information on the growth rate of this species in the first year. An alternative hypothesis is that the initial mode we found is of 2-y-old abalone. We discount this possibility because of known laboratory growth rates in the first year cited above, and also because we could detect animals down to at least 10 mm SL on crustose corallines. If another mode were present, we should have seen it.

Von Bertalanffy growth parameters for Alaskan abalone are remarkably close to those in British Columbia. Sloan and Breen (1988) reviewed growth at nine sites in British Columbia. Excluding the values for Lyell Island because there were few tag recoveries there, they recorded for eight sites a mean K value of 0.24 (range 0.16–0.24) and a mean L_∞ value of 120.1 mm (range 95.2–137.3 mm) compared with our mean K value of 0.20 and L_∞ value of 126.7 mm for seven sites (Table 4). While the two sets of values are not strictly comparable because the values of Sloan and Breen are derived from length-increment data, whereas ours are from length-at-age data (see review of Day and Fleming 1992), they clearly show close similarity.

What is the cause of the differences in growth between sites? Breen (1980) considered that the growth rate of the pinto abalone was related to the food supply. Abalone in sheltered to semiexposed habitat in *Macrocystis* or *Nereocystis* forests grew faster and to a larger size than those in habitats dominated by the unpalatable kelp *Pterygophora* and exposed to ocean swell. All of our sites were in moderately to highly sheltered habitats, usually in places of moderate tidal current. The habitats were variously dominated by *Macrocystis*, *Nereocystis*, *Agarum* and *Laminaria* cf *saccharina*. Grant Cove at the northern end of Gravina Island was exposed to local seas to the north, and Jumbo Island, far up Hetta Inlet, was the most sheltered. There was little variation in growth between our sites, and we attribute this to the overall similarity of habitat.

Ring counting is an appealing alternative to tag-and-recapture methods for obtaining growth rates, which are important for regulating fisheries (Troynikov and Gorfine 1998). Ring counting is cost-effective, requiring only one sampling event, and does not negatively affect growth as tags may (reviewed by Day and Fleming 1992). Ring counting also provides length-at-age data, and not simply increments at size, and, with catch-curve analysis can provide estimates of the total mortality rate of a population. The present application has provided the first broad geographic depiction of abalone growth rates in Alaska. These data are an important component of the stock assessment program that must precede any future reopening of the Alaskan fishery after its collapse in the past 2 decades (Woodby et al. in press).

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PREDATION OF THE INVASIVE FRESHWATER MUSSEL *LIMNOPERNA FORTUNEI* (DUNKER, 1857) (MYTILIDAE) BY THE FISH *LEPORINUS OBTUSIDENS* VALENCIENNES, 1846 (ANOSTOMIDAE) IN THE RIO DE LA PLATA, ARGENTINA

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ABSTRACT A study of the presence of the freshwater mussel *Limnoperna fortunei* in the diet of the native fish *Leporinus obtusidens* was performed in Costanera Norte, Buenos Aires. Fish were collected monthly for a 1-year period to analyze their digestive tracts. From a total of 157 fish collected (20–55 cm total length), 21 had empty digestive tracts. Of the remaining 136 individuals, 98 (72.1%) contained fragments of *Limnoperna* shells. The mussel represented 14.5% of the stomach content dry weight and 44.4% of the intestinal content dry weight. *Limnoperna* was present in almost all fish over 30 cm (total length). The largest number of mussels ingested by a *Leporinus* individual was 77, estimated by mussel beak count. Mussels 15–20 mm in shell length to be the most abundant in *Leporinus* digestive tract.

KEY WORDS: Invasive mussel, *Limnoperna* control, fish predation

INTRODUCTION

Limnoperna fortunei(Dunker 1857) arrived in Río de la Plata, Argentina, in the early 1990s (Pastorino et al. 1993). The mussel attaches to any available hard substrate using byssal threads and can form dense aggregations. The rapid expansion and the high densities achieved in the Rio de la Plata and the Paraná basin (80.000 individuals per square meter recorded in 1993, Darrigran and Pastorino 1995; and more than 100.000 in 1995, Darrigran et al. 1998) give the impression that *L. fortunei* has not encountered local competitors, predators, or parasites.

A counter example is the Zebra mussel, *Dreissena polymorpha* (Pallas 1754), which invaded North America in the mid- 1980s. In laboratory conditions small Zebra mussels (< 8 mm in shell length) are preyed upon by Crayfish (Love and Savino 1993, MacIsaac 1994), and by the blue crab in estuarine conditions (Molloy et al. 1994). Several fish have been reported as significant predators of the Zebra mussel (French and Bur 1993, Marsden 1997, Tucker et al. 1996).

In Argentina, Darrigran and Colauti (1994) reported the importance of the native fish *Pterodoras granulosus* (Valenciennes 1833) as a predator on *Corbicula fluminea* (Müller 1774), another invasive freshwater species.

To identify possible predators of *Limnoperna fortunei* in the Río de la Plata, an analysis of fish gut contents caught in Costanera Norte, Buenos Aires was performed. Preliminary results showed that native *Leporinus obtusidens*, as well as being the dominant catch was a relevant predator of *Limnoperna fortunei* (Penchaszadeh et al. 1998). This paper is a specific study of the presence of the *L. fortunei* in the diet of *L. obtusidens*.

Leporinus obtusidens ("boga" is the common local name) has a small conic head, rounded snout, well-developed lips, and small frontal teeth in the mouth. Each maxillary has a single row of chisel-shaped teeth pointing forward, the two anterior being longer than the others: the name of the genus refers to this characteristic, this kind of dentition is reminiscent of leporinid rodents.

MATERIAL AND METHODS

Fish were collected form sports fishermen from March 1998 to February 1999 in Costanera Norte, Rio de la Plata, 34°34′S, 58°23′W; a location heavily colonized by *Limnoperna*. The tidal regime is mixed, although predominantly semidiurnal with a tidal range of 0.63 to 1.07 m. Water levels and currents are strongly influenced by meteorological conditions, mainly wind direction and intensity, the most important of which is the "Sudestada." This is characterized by a gradual and persistent increase in wind speed blowing from the SE to the SSE, during which time, winds can pick up to around 25 m/sec (Guerrero et al. 1997). Heavy rain in the Parana basin can also strongly influence water levels. Mean surface water temperature values for Costanera Norte are: summer (January to March) 24.07 ± 2.01 °C; fall (April to June) 13.83 ± 4.68; winter (July to September) 14.63 ± 2.00 and spring (October to December) 21.04 ± 0.11.

Fish length was measured to the nearest 1 cm and then dissected *in situ*. The entire digestive tract was removed and preserved in a 10% formalin solution. In the laboratory, stomach and intestine contents of each *Leporinus* were analyzed under a dissecting microscope and separated into *Limnoperna* shells and other material. Then, the dry weight (80 °C until constant weight was achieved) of the two groups was taken.

Limnoperna beaks (anterior portion of the valve containing the umbonal region) were counted, the total number then divided by two was the estimated number of *Limnoperna* contained in each fish. To estimate the length of the mussels consumed, *Limnoperna* individuals were collected from the same fishing site in January, 1999. These mussels were then measured and separated into four size ranges. Ten mussels from each category were weighed, and a proportion of shell length to shell weight was obtained for each category. The total shell weight in the digestive tract of each fish was then divided by the number of *Limnoperna* eaten, estimated by the beak count.

RESULTS

Of the 157 *Leporinus* analyzed, the digestive tracts of 21 were empty. Of the remaining 136 individuals, 98 (72.1%) had fragments of *Limnoperna* shells in the stomach, the intestine, or both (Table 1). *Limnoperna* shells represented 14.5% of the stomach content dry weight and 44.4% of the intestinal content dry weight. Considering the entire digestive tract, 33.4% of the content dry weight was *Limnoperna* shells. The digestive tracts did not show damage or bleeding, although they were sometimes fully packed with shell fragments.

Limnoperna was present in the diet of *Leporinus* during almost the entire year of sampling. Lowest values occurred during winter (June–August) when mussel shells were found in only 40, 14, and 0% of analyzed fish, respectively (Table 1). Winter was also the season of lowest *Leporinus* catch in the study area.

Except for six individuals, *Limnoperna* was present in all fish (47) over 30 cm (total length) with material in their digestive tract. The presence of *Limnoperna* in the *Leporinus* digestive tract was maximum during the period from October to February (83 to 100%).

The largest number of mussels ingested by a *Leporinus* individual was 77 (beak count method). According to the shell length–weight proportion found (Table 2), the 10 fish with the largest number of ingested mussels contained various sizes of prey. The mean shell weight ranged between 0.012–0.020g (<10 mm of shell length, 20%), 0.03–0.068g (10–15 mm of shell length; 70%) and 0.175 g (15–20 mm of shell length; 10%). These results indicate that *Limnoperna* of between 10–15 mm in shell length were the most abundant in *Leporinus* digestive tract.

In 36 cases, entire *Limnoperna* individuals with unbroken shells and intact soft tissue were found in the digestive tract of *Leporinus*. Mussels measured between 1.5 and 5.7 mm in shell length and fish ranged 26.0 to 33.0 cm (total length).

Aquarium observations (M. Brögger, pers. comm.) show that *Leporinus* do not always remove the entire mussel from its substrate; on several occasions fragmented mussels with the beak area still attached to the substrate were observed. Other mussels >30 mm had bite marks on the periostracum.

DISCUSSION

Before the *Limnoperna* invasion there were few published reports on *Leporinus obtusidens* diet, and almost all of these are confined to the middle Parana river region (Mastrarrigo 1950, De Occhi and Oliveros 1974).

Mastrarrigo (1950) defined the alimentary regime of *Leporinus* as mainly omnivorous, with a high proportion of aquatic vegetation (in the case of at least 72% of the fish analyzed in Rosario, middle Paraná), but also mentioned the presence of a small proportion of fragmented river snails in the gut. Snails were referred to as the main food for *Leporinus* in the Uruguay river (Gualeguaychú, Entre Ríos), where their digestive tracts are often full of shell fragments. Mastrarrigo (1950), commented that local fishermen called them "bogas caracoleras" ("snail-eating bogas").

According to De Occhi and Oliveros (1974), mollusks were present in 32% of the examined *Leporinus* (5% bivalves and 27% gastropods, basically *Helobia* sp.; however, seeds and fruits were always dominant (37%). They believe that in *Leporinus obtusidens* because of the disposition of the pharyngeal teeth, these are used only to crumble soft material, because they lack crushing surfaces; whereas, fragmentation is achieved by action of the oral teeth.

Aquarium observations show that *Leporinus* did not always ingest the entire prey; instead, they bit off only a portion of the mussel (the bivalve was not completely removed from the substrate on many occasions). These observations suggest that there could be a underestimation in the amount of ingested mussels calculated by the beak counting method. This also could lead to an overestimation in the calculated size of ingested mussels.

The weight of *Limnoperna* in the fish digestive tracts could also be underestimated, because the soft material was weighed as a whole (with certain contribution of mussel tissue).

Differences observed in the amount of *Limnoperna* shell found in the stomach content (14.5%) and intestine content (44.0%) could indicate that *Leporinus* is preferentially an early–morning feeder, because all of the studied material was captured between 12:00 m. and 5:00 p.m.

The most abundant *Limnoperna* sizes in *Leporinus* digestive tracts were 10–15 mm in length. According to Boltovskoy and Cataldo (in press), who estimated *Limnoperna* growth in experi-

TABLE 1.

Presence of the freshwater mussel *Limnoperna fortunei* in the digestive tract of the fish *Leporinus obtusidens* in Costanera Norte, Buenos Aires (1998–1999).

Month	n	Fish Length (cm)			Digestive Tract with Contents (%)	Presence of *L. fortunei* in the Contents (%)
		Mean	SD	Range		
March	14	26.8	3.6	20–33	92.86	69.23
April	13	28.9	8.6	20–55	76.9	60
May	41	26.5	5.1	10.5–43	75.6	70.96
June	6	27.1	3.2	21–30	83.4	40
July	8	26.5	1.7	24–29	87.5	14.28
August	2	27.5	4.9	24–31	50	0.0
September	16	28.6	4.0	22–37	86.7	53.85
October	8	31.6	2.7	28–35	87.5	100
November	7	27.1	5.4	17–33.5	85.7	83.3
December	18	30.8	4.2	22.5–37	100	94.4
January	17	31.8	4.4	24–39	100	88.2
February	7	27.9	4.1	20–33	100	85.7
Total	157	28.4	5.1	10.5–55	86.6	72.1

TABLE 2.

Proportion of shell length to shell weight for four size-range groups of *Limnoperna fortunei*.

Shell length (mm) (anterior to posterior)	5–10	10–15	15–20	20–25
Shell weight (g)	0.022	0.067	0.172	0.317

mental conditions. these sizes correspond to mussels 3 to 6 months old.

The presence of *Limnoperna*, with its extraordinary abundance, occupying a seemingly empty niche in the Plata basin, has introduced a new element in the diet of some fish and constitutes the main food item for *Leporinus obtusidens*. Although other predators have yet to be identified, the "boga" has proved to be an important natural enemy of the invading mussel, *Limnoperna fortunei*.

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EMERSION AND THERMAL TOLERANCES OF THREE SPECIES OF UNIONID MUSSELS: SURVIVAL AND BEHAVIORAL EFFECTS

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ABSTRACT We evaluated the behavior and survival of unionid mussels after emersion in air temperatures across a range that is likely to be encountered during status surveys or relocations. Five laboratory tests were performed with pocketbook *Lampsilis cardium* Rafinesque (2 tests), pimpleback *Quadrula pustulosa* Lea (1 test), and spike *Elliptio dilatata* Rafinesque (2 tests) mussels, each conducted in a completely randomized, nested experimental design. For each mussel species (except *Q. pustulosa*), treatments tested included two water temperatures (25 and 10 °C), five air temperatures (ranging within ±20 °C of the water temperature), three aerial exposure durations (15, 30, and 60 min), and a no emersion control. All treatments were duplicated, with 10 organisms per emersion time and aerial exposure temperature (n = 320 mussels per test). Behavioral response (ability to upright) and mortality were measured daily for 14 d postemersion. Both water and aerial exposure temperature (air shock) were important predictors of times to first uprighting. The intensity function of first uprighting differed among species ($P < 0.01$), and there was a significant interaction between *E. dilatata* versus the other species and water temperature ($P < 0.01$). Over-all mussel survival after emersion was high (93%); however, *E. dilatata* experienced significant treatment related mortality at the 25 °C test water, 45 °C aerial exposure temperature. Because of the high incidence of uprighting and survival of mussels in our study, emersion at moderate temperatures (15 to 35 °C) and durations (15 to 60 min) does not seem harmful to mussels, and, therefore, conducting relocations and status surveys under these conditions should not impair mussel survival and over-all success.

KEY WORDS: Unionid mussel, conservation, emersion, temperature, behavior, mortality

INTRODUCTION

The imperiled status of unionid mussels (Williams et al. 1993) has prompted conservation efforts by public and private natural resource agencies that include status surveys, restocking, and relocation. The effects of collection and handling on mussels in field studies are generally considered benign and inconsequential to mussels relative to most threats (construction, zebra mussel infestation, habitat loss). However, Cope and Waller (1995) reviewed the success of relocation projects and found that mortality of mussels after relocation can be significant (>70% in 30% of projects reviewed). Mortality was highest within 1 year of the event, suggesting that effects of collection, handling, and displacement of mussels may be greater than were previously considered. The environmental conditions that mussels experience during collections and surveys may contribute to low survival, but can also be controlled to some extent. Determination of the emersion and thermal tolerances of unionid mussels would provide guidelines on the conditions in which surveys and relocations should occur to enhance mussel survival and over-all success.

Past studies suggest that mussels can tolerate emersion for hours or even days (Byrne and McMahon 1994, Dietz 1974, Holland 1991, Schanzle and Kruze 1994, Waller et al. 1995). However, survival of mussels is related to such environmental conditions during emersion as relative humidity and air temperature. For example, Waller et al. (1995) emersed *Amblema plicata plicata* Say and *Obliquaria reflexa* Rafinesque for a maximum of 8 h and found that mussels had greater survival when handled during the fall (water temperature ~15 °C; air temperatures ranged from 12 to 25 °C) compared to those handled during the spring (water temperature ~23 °C; air temperature ranged from 18 to 29 °C). In the present laboratory study, we augment these data by evaluating a range of extreme air temperatures and water–air thermal differentials. We selected the minimum and maximum water and air temperature and emersion times based on conditions likely to be found in field collecting situations. In addition to survival, the uprighting behavior of mussels after emersion was selected as a potential indicator of emersion stress; presumably, the ability to upright and burrow into the substratum indicates normal functioning. Waller et al. (1999) found significant species and water temperature related differences in the uprighting and movement intensity of four mussel species after displacement. Thus, displacement, coupled with a thermal and emersion challenge, may also produce significant behavioral changes.

In this study, we evaluated the effects of emersion and temperature on the survival and behavior of three mussel species *Lampsilis cardium* Rafinesque (pocketbook), *Quadrula pustulosa* Lea (pimpleback), and *Elliptio dilatata* Rafinesque (spike), and examined the variation in survival and behavioral response within and among the three species. These mussel species represent two subfamilies (Lampsilinae and Ambleminae) and two contrasting life history strategies (long-term and short-term brooders) within the Unionidae. Additionally, *L. cardium* and *Q. pustulosa* served as surrogates for two U.S. Federally Endangered species, the *L.*

higginsi Lea (Higgins' eye) and *Q. fragosa* Conrad (winged mapleleaf), both found in the Upper Mississippi River basin. *Elliptio dilatata* was chosen as a second surrogate for *Q. fragosa*, because too few *Q. pustulosa* were available for testing at low (10 °C) water temperature.

MATERIALS AND METHODS

Test Organisms

Three species of unionid mussels were collected from the Wolf River at Shawano, Shawano County, Wisconsin. Mussels were transported in holding tanks, containing Wolf River water (25 °C), to the Upper Midwest Environmental Sciences Center, in La Crosse, Wisconsin. Holding tank water temperatures were maintained at 25 ± 3 °C (with addition of nonchlorinated ice as needed), and the dissolved oxygen concentration was maintained at >60% saturation with aeration. Water temperature and dissolved oxygen (Yellow Springs Instrument Model 58 oxygen meter) were measured at 1-h intervals. At the laboratory, mussels were placed into submerged cages held in the Black River (water temperature, 27 °C), near La Crosse, Wisconsin until study initiation. The mussel cages (122-cm length × 122-cm wide × 46-cm height) were constructed of angle and strap iron frame with netting (1.9-cm diam. polyethylene) attached to the iron frame by tie wraps and nylon rope. One species of mussel (111 total; density of 75/m^2) was placed into each cage. During collection, transport, and allocation to cages, mussels were continually immersed in river water.

Experimental Design and Exposure System

Five laboratory tests were performed with *L. cardium* (2 tests), *Q. pustulosa* (1 test), and *E. dilatata* (2 tests), each conducted in a completely randomized design as a nested experiment. For each mussel species tested (except *Q. pustulosa*), there were two water temperature treatments (25 and 10 °C), five air temperatures (ranging within ± 20 °C of the water temperature), three aerial exposure duration treatments (15, 30, and 60 min), and a no emersion control treatment (Fig. 1). Because of limited availability, *Q. pustulosa* was tested only at 25 °C, the treatment we assumed to be more lethal. All treatments were duplicated, with 10 organisms per emersion time and temperature (n = 320 mussels/test), for a total of 32 experimental units. Ten mussels were placed into a flow-through, stainless steel tank (61-cm length × 30-cm wide × 36-cm height) containing sand (13 ± 0.5-cm depth) and 42 ± 0.5 L of overlying well water. Each tank was placed into one of six water baths (305-cm length × 84-cm wide × 46-cm height) maintained at the test temperature (10 or 25 ± 1 °C) with a thermostatically controlled, liquid circulation pump (Remcor Model CFF-501, Remcor Products Co., Franklin Park, IL) connected to the water bath. Tanks were aerated to maintain dissolved oxygen concentrations at >60% saturation. The flow rate of water into each tank was 200 mL/min with a turnover rate of seven times per day. The photoperiod was 16-h light and 8-h dark.

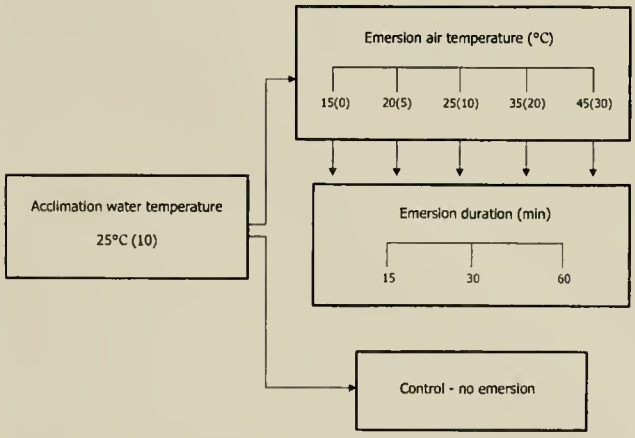

Figure 1. Experimental design for the thermal and emersion exposure tests with three species of unionids. The numbers in parentheses represent the 10 °C test water–aerial exposure regime.

Laboratory tests began when the water temperature of the Black River reached the desired test water temperature (25 °C; July/August and 10 °C; November/December). Each mussel species was tested individually, and a given species was transported in coolers containing Black River water to the laboratory for testing. Ten mussels were randomly selected for each experimental unit. Plastic mesh netting was placed on top of the sand substrate to prevent mussels from burrowing into the substrate before aerial exposure. Mussels were acclimated in their respective tanks at a water temperature of 25 ± 1 °C for 2 d; mussels were not fed during the acclimation period. Mussels within a replicate were numerically marked (1 to 10) on their right valve with a permanent marker. To enable identification of mussels after burrowing into the substrate, each mussel was uniquely tagged with a numbered fishing bobber (3.81-mm dia.) that was attached to a 22.9-cm piece of cotton thread and secured to the umbonal region of the right valve with cyanoacrylate (Krazy Glue® Gel, Borden, Inc., Columbus, OH). Both siphons (incurrent and excurrent) remained immersed while bobbers were being attached to the shells; total handling time was less than 3 min per mussel.

For each treatment, 20 mussels (10 from each replicate) were removed from the test water (25 or 10 °C), transported in water (held at test temperature), and placed into an environmental chamber (Hotpack® Biological Chamber, Hotpack Corp., Philadelphia, PA) at a given air temperature (25 °C water temperature; 15, 20, 25, 35, 45 °C air temperature; 10 °C water temperature; 0, 5, 10, 20, 30 °C air temperature) for a duration of 15, 30, or 60 min. Treatments were conducted in order of increasing air temperature and emersion duration. The target relative humidity in the environmental chambers was 60 ± 5%. This relative humidity was selected based on average ambient air conditions experienced in our geographic region (Steve Thompson, National Oceanic Atmospheric Administration, La Crosse, WI, pers. comm.). Following emersion, mussels were removed from the environmental chamber, transported in well water (held at the test temperature, 25 or 10 °C), and returned to their respective tanks. Each mussel was placed directly on top of the sand substrate, with the right valve (tagged side) facing upward. Test organisms were fed a mixture of C4 algae diet (Coast Seafoods Co., South Bend, WA; 0.2 mL per mussel) and dry *Chorella* (0.013 g dry weight per mussel) daily. Mussels were monitored for mortality and uprighting response for 14 d postemersion. At test termination (14 d postemersion), mussels were recovered from each tank and measured for total length and whole mussel wet weight. Sex of *L. cardium* was determined by shell dimorphism. *Elliptio dilatata* and *Q. pustulosa* are not sexually dimorphic; thus, we examined histological sections of half of the mussels from each replicate in the 25 °C test to determine the sex ratio, and assumed animals tested at the 10 °C water temperature had a similar sex ratio, because all mussels came from the same population and were randomly sampled.

Statistical Analyses

For each mussel species, we examined patterns between two response variables, times to first uprighting and death, as a function of water temperature (°C), duration of aerial exposure, and air shock temperature (°C), which we define as the difference between water temperature and air emersion temperature. We refer to both first uprighting and death as events, and our primary data consist of elapsed times to occurrences of those events for each mussel. Some event durations may have exceeded the study duration (14 d) and, therefore, went unobserved; these events are said to be "right-censored" (Hosmer and Lemeshow 1999). Proper accommodation of censoring is critical to valid interpretation of time-to-event data. For both events (first uprighting and death), we used the Cox proportional hazards regression model (Cox 1972, Newman 1995, Hosmer and Lemeshow 1999) to identify factors that explained the pattern in uprighting and survival. We arbitrarily selected *L. cardium* as the baseline species for our analysis; this choice does not affect the over-all results. The baseline temperature was 0 °C, and other temperatures were coded as deviations from this baseline. Denote E = U and E = D for the events uprighting and survival, respectively. Our full regression models for both first uprighting and survival are

$$\lambda_{Ei}(t) = \lambda_{E0}(t)\exp(S_i + \beta_1 T + \beta_2 A + \beta_3 M + \beta_4 A^2 + \beta_5 M^2 + \beta_{1,i} T + \beta_{2,i} A + \beta_{3,i} M + \beta_6 TA + \beta_7 TM + \beta_8 AM + \beta_9 TA^2 + \beta_{4,i} A^2 + \beta_{6,i} TA + \beta_{7,i} TM + \beta_{8,i} AM + \beta_{11} TAM + \beta_{11,i} TAM), \quad (1)$$

where: $\lambda_{Ei}(t)$ is the hazard function for event type E for the ith species at time t; $\lambda_{E0}(t)$ is the corresponding baseline hazard; $S_i, i = 1, 2$, are two fixed-effects parameters for identification of the three species (S_1 denotes *E. dilatata*, and S_2 denotes *Q. pustulosa*); T represents water temperature (°C) with coefficient β_1; A represents air shock temperature (°C), which we define as the difference between water and air emersion temperatures with coefficient β_2; M represents air exposure duration (min) with coefficient β_3; A^2 and M^2 are quadratic (∪ or ∩-shaped) effects of air shock and exposure duration, respectively; $\beta_{1,i}T$ represents the species × water temperature interaction; $\beta_{2,i}A$ represents the species × air shock interaction; $\beta_{3,i}M$ represents the species × air-exposure duration interaction; TA, TM, and AM are two-way interactions among T, A and M; TA^2 is the interaction between T, and the quadratic effect of A; $\beta_{4,i}A^2$ is the interaction between species and A; $\beta_{6,i}TA$, $\beta_{7,i}TM$, and $\beta_{8,i}AM$ represent three-way interactions among species, A, T, and M; TAM is the three-way interaction between T, A, and M; and $\beta_{11,i}TAM$ is the four-way interaction among species, T, A, and M. In this model, "interactions" are on the log scale. Although in such terminal events as death, it is customary to refer to $\lambda(t)$ as the hazard function for deleterious events such as death, the term intensity is more appropriate than hazard for events such as first uprighting. Therefore, we refer to $\lambda(t)$ as either the hazard or intensity function, depending on whether we are addressing survival or first uprighting, respectively. We fitted Eq. (1) to the uprighting and survival data by maximizing the partial likelihood (Cox 1972), and constructed likelihood-ratio and Wald chi-square tests for each parameter (Hosmer and Lemeshow 1999) with the SAS PHREG software (SAS Institute 1997). For each event type, we began with our full regression model [Eq. (1)] and, one-by-one, deleted terms for which the corresponding likelihood-ratio chi-square test was not significant at the $\alpha = 0.05$ level, except we did not delete terms for which a higher-order interaction was statistically significant. This model reduction process identifies the simplest model for each event type that preserves the hierarchical structure of Eq. (1). Our recorded event times were based on observations at fixed times rather than exact measurements of event times, and, therefore, contained ties. We used Efron's method to adjust for tied event times, which has been shown to perform better than alternatives (Hertz-Picciotto and Rockhill 1997). We assumed that events occurred at the observation time rather than, for example, the temporal midpoint between successive observations to produce conservative estimates of the intensity of first uprighting or the hazard of mortality.

A particularly desirable feature of proportional hazard regression models is that the parameters have natural interpretations that provide informative descriptions of the event times. Because these models are still unfamiliar in ecology, interpretation requires some explanation. The hazard function $\lambda(t)$ quantifies the number of events per interval of time at time t. From Eq. (1), the dimensionless hazard ratio (risk ratio) at time t is given by $HR(t) = \lambda_i(t)/\lambda_0(t) = \exp[S_i + \cdots + \beta_{11,i}TAM]$. For such categorical variables as species S_i in our analysis, the hazard ratio for species i relative to the baseline species is $\exp(S_i)$, and the hazard ratio for species 1 relative to species 2 is $\exp[S_1 - S_2]$ in the absence of higher-order interactions. If, for example, $\exp(S_i) = 0.5$, we say that the *relative* hazard (or intensity) for species i is only 50% of that for the baseline species. For continuous covariates such as water temperature, the statistic $100[\exp(\beta_1) - 1]$ is the estimated percentage change in the hazard (or intensity) ratio for each unit change in temperature. If, for mortality, $\beta_1 = 0.5$, then the hazard ratio increases by approximately 65% for each 1 °C increase in temperature (Allison 1995). These interpretations extend to more com-

TABLE 1.

Physical characteristics of three mussel species after aerial exposure at various water–air temperature differentials.

Water Temperature (°C)	Species	Mean Length (mm)	Wet Weight (g)
25	*Elliptio dilatata*	80.57 (9.7)	53.79 (18.4)
	Quadrula pustulosa	61.82 (13.2)	78.27 (42.0)
	Lampsilis cardium	100.32 (10.7)	185.59 (54.0)
10	*E. dilatata*	78.64 (10.4)	52.21 (19.6)
	L. cardium	99.71 (11.6)	179.74 (33.1)

Numbers in parentheses are the standard deviation of the mean.

TABLE 2.

Proportional hazards (intensity) model fitted to the time to first uprighting for *Elliptio dilatata*, *Quadrula pustulosa*, and *Lampsilis cardium*. *Lampsilis cardium* at 0 °C constitute baseline conditions.

Parameter (Effect)	Estimate (SE)	Wald χ^2	*P*-Value
S_1 (species: *E. dilatata*)	0.3142 (0.1965)	2.6	0.11
S_2 (species: *Q. pustulosa*)	− 0.3031 (0.0821)	13.6	< 0.01
β_1 (water temperature T)	0.1491 (0.0083)	319.0	< 0.01
β_2 (air shock A)	− 0.0677 (0.0146)	31.4	< 0.01
β_4 (A^2)	0.0055 (0.0011)	26.9	< 0.01
$\beta_{1,1}$ ($S_1 \times T$)	− 0.0562 (0.0096)	34.1	< 0.01
β_6 ($T \times A$)	0.0027 (0.0007)	16.2	< 0.01
β_9 ($T \times A^2$)	− 0.0003 (0.0001)	30.9	< 0.01

See text for explanation of the model and parameters.

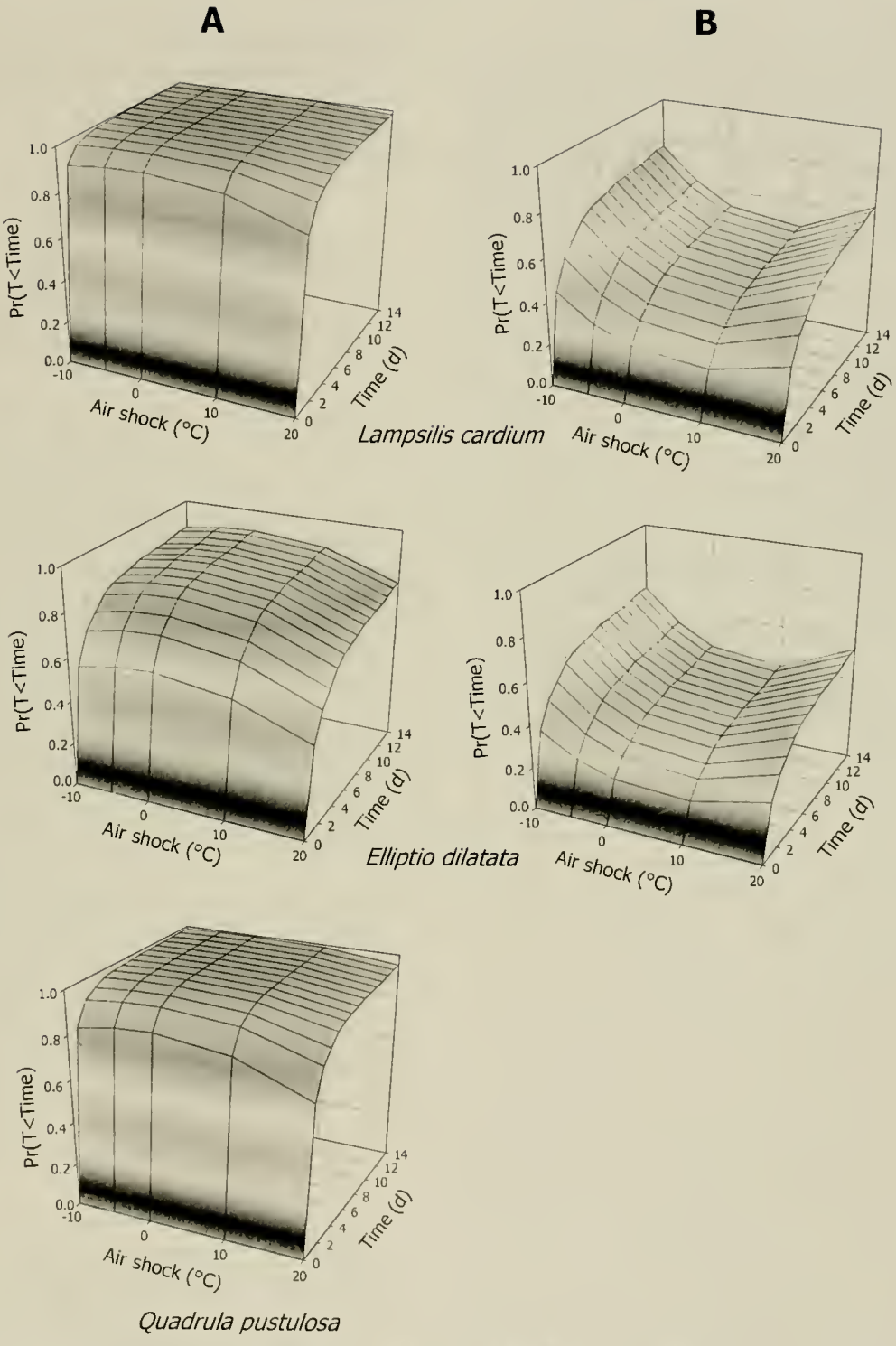

Figure 2. Estimated probabilities of times to first uprighting, T, were less than times on the axis marked Time for *L. cardium*, *E. dilatata*, and *Q. pustulosa* at five air shock temperatures obtained from proportional hazards model (Table 2). Air shock was defined as the difference between water and air emersion temperatures. Letters A and B refer to the 25 and 10 °C water exposure treatments, respectively.

plicated models having significant interactions. For example, from Eq. (1) the hazard ratio for species 1 to species 2 at water temperature T, air shock temperature A, and exposure duration M is given by $\exp[S_1 - S_2 + (\beta_{1,1} - \beta_{1,2})T + (\beta_{2,1} - \beta_{2,2})A + (\beta_{3,1} - \beta_{3,2})M + (\beta_{4,1} - \beta_{4,2})A^2 + (\beta_{6,1} - \beta_{6,2})TA + (\beta_{7,1} - \beta_{7,2})TM + (\beta_{8,1} - \beta_{8,2})AM + (\beta_{11,1} - \beta_{11,2})TAM]$.

Although hazard ratios have natural interpretations that provide the means to assess the relative importance of the explanatory variables, we display model features using graphs of the "survivor" functions (Hosmer and Lemeshow 1999), which are probabilities that times until events exceed some time t. We computed product-limit survival estimates (Kalbfleisch and Prentice 1980) of the

survivor function and used those to display model features for both first uprightings and deaths.

RESULTS

Physiochemical Characteristics of Water

Temperature, dissolved oxygen (Yellow Springs Instrument Model 58 oxygen meter), and pH (Beckman Model Φ11 meter) were measured daily in each tank during each test. Averages and standard deviations (SD, in parentheses) for physiochemical characteristics of water in all tanks at each water temperature (25 and 10 °C, respectively) were as follows: temperature 24.5 °C (0.8), 10.1 (0.4); dissolved oxygen 8.2 mg/L (0.4), 11.5 mg/L (0.6); pH 8.10 (0.06), 8.13 (0.09). Un-ionized ammonia concentrations (mg/L) were measured in six randomly selected tanks for each test (range, 0.0013 to 0.0054 mg/L), and were well below the concentrations reported to adversely affect mussel growth (0.036 mg/L at 6 weeks, Sparks and Sandusky 1981) or survival (96 h LC50 = 1.1 mg/L, Arthur et al. 1987). The mean relative humidity over all five tests during aerial exposures was 63.6 (2.19).

Mussel Characteristics

The average length and wet weight of mussels were similar within a species between water temperatures (Table 1). The sex ratios (male:female) of *E. dilatata* and *Q. pustulosa* in the 25 °C treatment were similar (66 male:67 female and 74 male:65 female, respectively); however, the sex ratio for *L. cardium* was approximately 2:1 males to females (209 male:111 female) in the 25 °C water treatment and approximately 3:1 males to females (239 male:80 male) in the 10 °C water treatment.

Uprighting Behavior

The intensity of first uprighting differed among species, water temperatures, and air shock temperatures in a complex way involving multiple interactions (Wald $\chi^2 = 633.2$, $df = 8$, $P < 0.01$). There was a significant interaction between *E. dilatata* versus the other species and water temperature ($P < 0.01$; Table 2). Both water temperature and air shock temperature were important predictors of times to first uprighting. As expected, the intensity of uprighting was greater at the higher water temperature. Moreover, the effects of air shock differed with water temperature and showed a significant quadratic response (∪ or ∩-shaped, Fig. 2). At the 10 °C water temperature, the uprighting response was ∪-shaped; whereas, at the 25 °C water temperature, the response was ∩-shaped. Although air exposure duration had no statistically significant effect, it is important to note that, by definition, any response to air shock temperature requires exposure. In this experiment, the briefest air shock duration was apparently sufficient to affect uprighting intensity, and longer durations showed no additional effect.

In addition to uprighting, we also observed other behavioral responses to emersion. Shell gaping behavior was observed in *L. cardium* during emersion in >25 °C air for 30 min and in *E. dilatata* during emersion in 45 °C air for 15 min. Also, the occurrence of foot extension increased with emersion time in *E. dilatata* at 45 °C (~70% at 15 min duration to ~100% in the 60-min exposure duration). All three species extruded mucus from the siphonal region after emersion in 45 °C air for 60 min.

Survival

Survival of mussels differed among species and with water temperature, air shock, and air exposure time in a complicated way involving both two- and three-way interactions (Wald $\chi^2 = 253.82$, $df = 14$, $P < 0.01$). Survival of *Q. pustulosa* did not differ significantly from *L. cardium*, the baseline species, at any water or air shock temperature, or with air exposure time (Table 3, Fig. 3). *Elliptio dilatata* differed significantly from *L. cardium* up through interactions with the linear and quadratic effect of air shock temperature, and the three-way interaction among species, water temperature, and air shock temperature (Table 3). The parameters (effects) for *E. dilatata* (S_1), air shock (A), squared air shock (A^2), and the *E. dilatata* × air shock ($S_1 \times A$), water temperature × air shock ($T \times A$), air shock × exposure duration ($A \times M$), *E. dilatata* × squared air shock ($S_1 \times A^2$), *E. dilatata* × water temperature × air shock ($S_1 \times T \times A$), and water temperature × air shock × exposure duration interactions ($T \times A \times M$) were significantly different from zero (Table 3). Through the last day of the experiment, survival varied only slightly except for the *E. dilatata* in the 25 °C water temperature treatment that were exposed to large positive air shocks (Fig. 3). For *E. dilatata* in the 25 °C water treatment, survival probabilities decreased significantly in the 60-min air exposure duration treatments (Fig. 4).

DISCUSSION

Over-all mussel survival after emersion was high (93%) and indicated that these mussel species are remarkably resistant to emersion and thermal shock. For example, in the 10 °C water tests, both *L. cardium* and *E. dilatata* survived the air shock treatments despite a 20 °C air–water differential and emersion in subzero air. However, variations in tolerances to water–air treatments were evident among species at the higher water temperature. *Elliptio dilatata* died within 24-h postemersion to the 45 °C air temperature treatment, with 100% mortality at the 60-min aerial exposure duration and 50% mortality at the 30-min duration. Surprisingly, the other two species survived the highest air shock treatment. Several studies have documented that mussel survival, during and after emersion, is directly related to relative humidity (Byrne and Mc-

TABLE 3.

Final fitted proportional hazards model (equation 1) for *Elliptio dilatata*, *Quadrula pustulosa*, and *Lampsilis cardium* survival data. *Lampsilis cardium* at 0 °C constitute baseline conditions.

Parameter (effect)	Estimate (SE)	Wald χ^2	*P*-Value
S_1 (species: *E. dilatata*)	−1.8979 (0.8667)	4.8	0.02
S_2 (species: *Q. pustulosa*)	− 0.0261 (0.4375)	<0.1	0.95
β_1 (water temperature T)	−0.0352 (0.0433)	0.7	0.42
β_2 (air shock A)	0.2702 (0.0731)	13.7	< 0.01
β_3 (exposure duration M)	0.0266 (0.0163)	2.6	0.10
β_4 (A^2)	−0.0034 (0.0018)	3.7	0.05
$\beta_{1,1}$ ($S_1 \times T$)	0.0253 (0.0471)	0.3	0.59
$\beta_{2,1}$ ($S_1 \times A$)	−0.3527 (0.0633)	31.0	<0.01
β_6 ($T \times A$)	−0.0148 (0.0034)	18.4	< 0.01
β_7 ($T \times M$)	− 0.0004 (0.0009)	0.2	0.63
β_8 ($A \times M$)	−0.0042 (0.0014)	9.4	<0.01
$\beta_{4,1}$ ($S_1 \times A^2$)	0.0126 (0.0029)	19.2	<0.01
$\beta_{6,1}$ ($S_1 \times T \times A$)	0.0164 (0.0032)	25.8	<0.01
β_{11} ($T \times A \times M$)	0.0003 (0.0001)	17.1	<0.01

See text for explanation of the model and parameters.

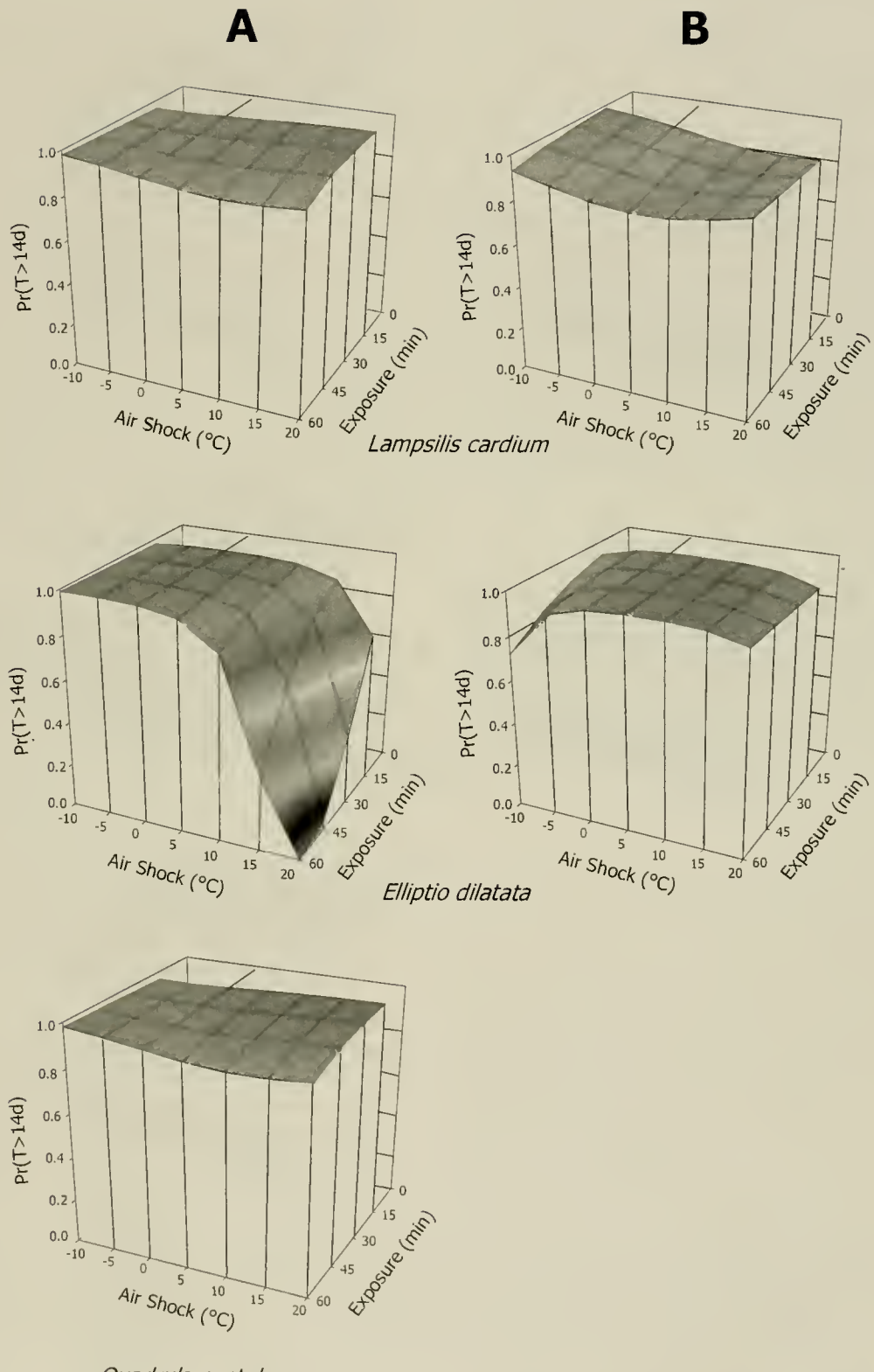

Figure 3. Estimated survival probabilities, T, were greater than times on the axis marked Time for *L. cardium*, *E. dilatata*, and *Q. pustulosa* at five air shock temperatures and three exposure durations obtained from proportional hazards model (Table 3). Air shock was defined as the difference between water and air emersion temperatures. Letters A and B refer to the 25 and 10 °C water exposure treatments, respectively.

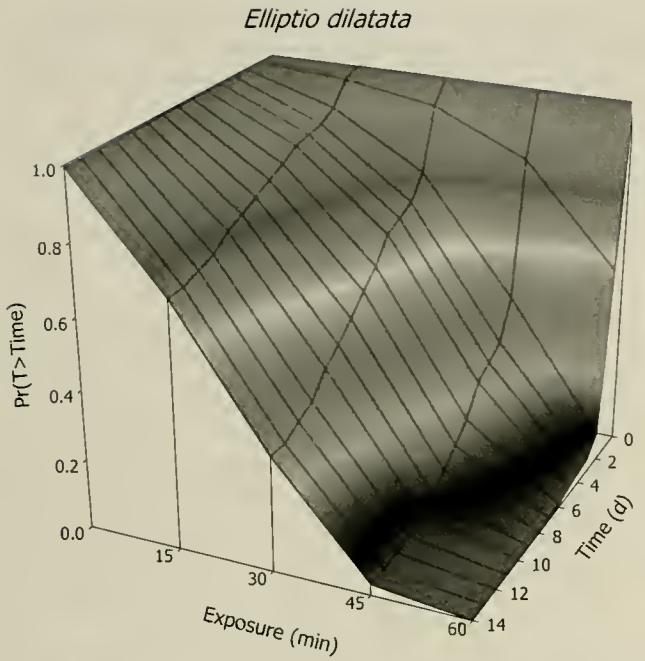

Figure 4. Estimated survival probabilities, T, were greater than time on the axis marked Time for *E. dilatata* at three exposure durations for the 25 °C water, 45 °C aerial emersion test obtained from proportional hazards model.

Mahon 1994, Dietz 1974, Holland 1991). Dietz (1974) reported that *Ligumia subrostrata* Say could survive >40 d in air, if water loss was retarded; however, survival was reduced to 6–10 d when mussels were exposed in air with a low relative humidity (45–55%) at 25 °C. Relative humidity was held constant (60 ± 5%) across all treatments in our study. Survival probabilities are expected to vary directly with relative humidity and should not be considered absolute.

Uprighting behavior was more sensitive to species and treatment effects than survival. *Lampsilis cardium* uprighted before *Q. pustulosa* (25 °C water test only) or *E. dilatata* (25 and 10 °C water tests) regardless of air shock exposure or duration. In addition, both *L. cardium* and *E. dilatata* required more time to upright when held at the cooler water temperature than those held at the warmer water temperature. Waller et al. (1999), similarly found that mussels displaced in cooler water temperatures (7 °C) required up to tenfold more time to upright than those displaced in higher water temperatures (21 °C). In addition, the uprighting intensity for *L. cardium* and *E. dilatata* in this study showed a significant quadratic response to air shock temperature (Fig. 2). This is particularly noteworthy, because at the lower water temperature (10 °C), air shocks that differed greatly from water temperatures in either direction seemed to stimulate uprighting; whereas, at the higher water temperatures (25 °C), large positive or negative air shocks tended to inhibit uprighting slightly.

In addition to the uprighting behavior, we made qualitative observations on other sublethal behavioral responses to emersion and thermal stress, which included shell gaping, foot extension, and mucus production. These behaviors were elicited during emersion in air >25 °C; whereas, mussels closed their valves in air temperatures between −10 to 20 °C. *Elliptio dilatata* exhibited all three responses (shell gaping, foot extension, and mucus production) at the higher air shock temperatures in contrast to partial responses (shell gaping and mucus production only) in both *L. cardium* and *Q. pustulosa*. There was an apparent correlation between the occurrence of these behaviors with survival. Byrne and McMahon (1994) reviewed emersion capacities of freshwater bivalves and found that dessication resistance was inversely related to the degree of mantle exposure behavior of a species. We suggest that these behavioral responses, along with uprighting, are valuable sublethal indicators of stress and could be further quantified.

Emersion tolerance in freshwater bivalves may also be affected by shell characteristics. For example, both *L. cardium* and *Q. pustulosa* are spherical in shape; whereas, *E. dilatata* is elliptical. A sphere has the largest volume per outside surface area of any geometrical configuration (Kreith 1973). Conversely, an ellipse has a larger surface area per volume, which may have allowed the *E. dilatata* to reach a higher internal temperature more quickly and for a longer duration than the two spherically shaped species. Other physical characteristics of the shell that may affect emersion tolerance are shell thickness and over-all shell size. Shell thickness was similar among the three species that we tested; *Q. pustulosa* had the thickest shell, followed by *E. dilatata* and *L. cardium*, which have comparable shell thickness. In general, we expect thick-shelled species, such as amblemines, to be most tolerant to emersion and thinner-shelled species, such as anodontines, to be least tolerant. In addition, smaller individuals of a given species will be more sensitive to emersion than larger individuals because of the larger surface area to volume ratio. The rank of the average size of mussels in this study was *E. dilatata* (smallest), *Q. pustulosa*, and *L. cardium* (largest). Thus, these additional physical factors, shell shape, thickness, and over-all size, may also explain the greater sensitivity of *E. dilatata* to emersion.

One of the primary objectives of this study was to establish temperature guidelines for collection and handling of two federally endangered species, *Q. fragosa* and *L. higginsi*. *Quadrula pustulosa* and *L. cardium* were chosen as potential surrogates, because they are congeners. *Elliptio dilatata* was chosen as a second congener of *Q. fragosa*, because it is also an amblemine, but was more abundant at the study site than *Q. pustulosa*. Surprisingly, *Q. pustulosa*, and *L. cardium*, although in different subfamilies, responded more similarly than *Q. pustulosa* and *E. dilatata*, the two amblemines. These results demonstrate the difficulties associated with the use of surrogates and extrapolation to other mussel species, particularly when there is no sound biological basis for their selection. Therefore, guidelines should err on the side of the most sensitive species for which data are available, because results are seldom validated with threatened and endangered species.

In conclusion, these data provide baseline information for developing management guidelines for handling the species tested. Generally, we found that these species were tolerant of emersion, but may experience mortality in extreme conditions. Our survival estimates are conservative, because they were derived from laboratory studies conducted under controlled conditions rather than under field conditions in which air temperature and relative humidity can vary significantly within several hours. In addition, the microclimate to which the mussels are exposed can be very different from measured air temperature and relative humidity. Mussel survival can be enhanced by conducting field work in moderate conditions (10 to 25 °C air temperature), protecting the mussels from extreme changes in air temperature, and covering them with damp cloths during emersion to maintain high relative humidity.

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ELEMENTAL SULFUR IN THE GILLS OF THE MANGROVE MUD CLAM *ANODONTIA EDENTULA* (FAMILY LUCINIDAE)

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ABSTRACT Different sizes of the mangrove mud clam *Anodontia edentula* were collected from the mangroves in Bgy. San Roque in Estancia, Iloilo, central Philippines, and the mantle, gill, and foot tissues were analyzed for elemental sulfur content. Mangrove mud (substrate) was also analyzed for total sulfur content to establish the possibility of clam–bacteria symbiosis in this lucinid clam. Sulfur analysis showed highly significant ($P < 0.0001$) amounts of elemental sulfur in the gills (247.64 ± 63.28 μmoles/g FW) compared with the quantities observed in the mantle (0.84 ± 0.22 μmoles/g FW). Elemental sulfur was absent from the foot tissues. Results also showed a significantly ($P < 0.05$) decreasing elemental sulfur from the newly collected clams (mean = 461.18 μmoles/g FW) compared to those reared in the laboratory (mean = 159.08 μmoles/g FW: with mangrove mud substrate; mean = 45.18 μmoles/g FW without substrate), which were analyzed weekly until week 3, indicating that stored elemental sulfur is being utilized by the bacteria in the absence of sulfide. Total sulfur content of mangrove mud *in situ* was higher than that used as substrate in the experiment; where there were no significant differences from initial to final readings. This shows that mangrove mud *in situ* is linked to a steady sulfur source.

KEY WORDS: *Anodontia edentula*, lucinid, gills, elemental sulfur

INTRODUCTION

The mangrove mud clam *Anodontia edentula* (Linné, 1758) is widely distributed in the Indo-West Pacific region (Poutiers 1998). In the Philippines, it is one of the popular bivalves harvested from sandy–muddy bottoms near mangrove areas (Poutiers 1998, Sotto and von Cosel 1982). It is one of the most noteworthy species and a highly prized delicacy in the coastal areas where it is abundant; hence, an important source of food and livelihood. It grows to a maximum size of 8–9 cm shell length (SL), total weight of 180–210 g and is a potential aquaculture species.

A. edentula belongs to order Veneroida, family Lucinidae (Poutiers 1998), together with most eulamellibranchs containing symbiotic bacteria (Schweimanns and Felbeck 1985).

Animal–bacteria symbioses in marine mollusks have been observed in hydrogen sulfide-rich habitats, such as anoxic basins, sewage outfalls (Felbeck et al. 1981), seagrass beds (Cavanaugh 1983), mangrove swamps (Frenkiel et al. 1996, Vetter 1985), and in organically rich sediments (Janssen 1992).

The presence of elemental sulfur in the gills of some clams as energy source for clam–bacteria symbiosis has been reported by Vetter (1985) for *Lucinoma annulata, Calyptogena elongata*, and *Lucina floridana* and Dando et al. (1985) for *Myrtea spinifera*. In a review of Somero et al. (1989), the gills of the bivalve *Calyptogena ponderosa* have the highest elemental sulfur content (2593.8 μmole/g); whereas, *Solemya reidi* gills have the lowest (15.8 μmole/g).

Symbiotic bacteria in Philippine bivalves were first discussed by Janssen (1992) after his observation of bacteria in the gills of *Codakia tigerina* and *A. edentula* (using light microscopy), and *Fimbria fimbriata* (using transmission electron microscopy). In the present study, the observation of deep brown to deep purple, thick and fleshy gills having a single demibranch on each side of the gonad of *A. edentula* agrees with the description of Allen (1958) for several genera of lucinids, Dando et al. (1985) for *M. spinifera* and Distel and Felbeck (1987) for *L. aequizonata, L. annulata* and *L. floridana*, all of which are known for endosymbionts. These typical characteristics of lucinid gills possessed by *A. edentula* may support the claims of Janssen (1992).

To support further the evidences mentioned above, this study aimed to establish sulfur-oxidizing metabolism in *A. edentula* by analyzing elemental sulfur content of its mantle, gill, and foot tissues.

MATERIALS AND METHODS

Collection of Samples

A. edentula specimens were obtained from a narrow band (10–15 m) of 15–20 ha mangrove area in Bgy. San Roque, Estancia, Iloilo in central Philippines (11°14′N, 123°8′E) (Fig. 1). The trees are mostly secondary growths of *Sonneratia* and *Avicennia* spp. During low tide of spring tide periods, a very wide tidal flat is exposed, reaching 200–250 m seaward from the mangrove forest. There is no freshwater input in the study area.

Clams were collected during the ebb of a spring tide at daytime with the help of a clam collector who can recognize the opening of the clams' anterior inhalant tube on the surface of the mud. A specialized gear made of flattened iron bar attached to a wooden handle was used in digging the substrate. With this, the tube that led to the clam was carefully traced. Depth of each clam from the surface was measured during collection.

Physicochemical parameters were monitored during clam collection. Temperature was measured with a mercury thermometer; salinity with an Atago refractometer; pH with WTW pH 192 meter; and dissolved oxygen with a YSI Model 51B DO meter. Total sulfur, water, and organic matter content of the sediment were determined as prescribed by Beaton et al. (1968) and Dando et al. (1985).

Analyses of Sulfur Content

The presence of elemental sulfur in the tissues of *A. edentula* was examined, because elemental sulfur is the by-product of sulfide oxidation carried out by symbiotic bacteria (Atlas 1995) and is stored in the periplasmic space (Vetter 1985). Total sulfur in the

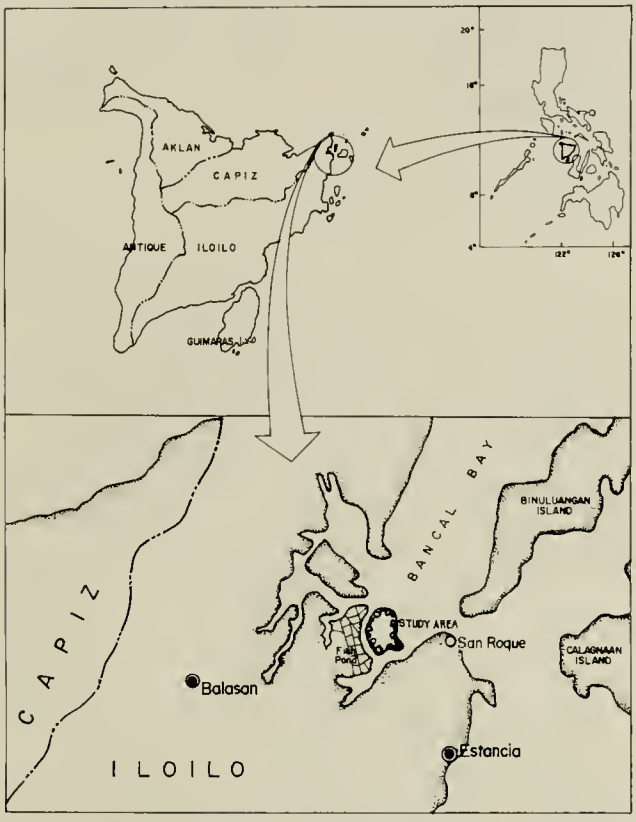

Figure 1. Map of Bgy. San Roque, Estancia, Iloilo in central Philippines showing location of collection site of *Anodontia edentula*.

substrate was measured both *in situ* and during laboratory experiments (weeks 1, 2, and 3) to know the levels in the clam's natural environment and the changes under laboratory conditions.

Elemental sulfur content of the tissues (mantle, gill, and foot) and total sulfur content of the substrate were determined initially (from newly collected samples) and through time (1–3 weeks) from laboratory-reared clams.

Clams and mangrove mud were collected from the study site and transported to the laboratory of the Aquaculture Department of Southeast Asian Fisheries Development Center (SEAFDEC/AQD) in Tigbauan, Iloilo. Upon arrival, all clams were measured (shell length, SL; shell width, SW; shell height, SH) using a caliper, weighed (total weight, TW) using a Mettler AE163 analytical balance, and numbered individually.

Elemental Sulfur Analysis

Six clams were randomly selected and dissected for their mantle, gills, and foot. Gill color of each clam was noted and described (Vetter 1985), and total wet weight and individual weights of each tissue were measured using a Mettler AE163 analytical balance. Tissues were then dried to constant weight in an oven at 60 °C, then pulverized by mortar and pestle. Elemental sulfur was extracted from the dried tissues using acetone and precipitated with barium sulfate seed suspension. Acidity of the medium (for sulfur extraction) was maintained by the addition of barium chloride (Beaton et al. 1968). Extracts were then subjected to turbidimetry. Absorbance was measured in a Shimadzu UV-1601 spectrophotometer at 440 nm wavelength. Elemental sulfur was computed and expressed as μmoles per g fresh weight (μmoles/g FW).

For comparison purposes, mantle, gill, and foot tissues of newly collected *Anadara antiquata*—another mangrove-associated bivalve inhabiting the same habitat as *A. edentula*—were also analyzed for elemental sulfur.

Two treatments (with and without mud substrate) replicated three times were prepared for sulfur analyses (elemental for the tissues and total for the substrate) through time. For the treatment with mud, three glass aquaria (0.75 × 0.44 × 0.42 m) were provided with 0.15 m newly collected mangrove mud to provide a substrate close to that found in their natural environment (Distel and Felbeck 1987, Vetter 1985), 100-L seawater (salinity conditions the same as in the field, 33–35 ppt), air stones, and 2-mm mesh black net cover. For the other treatment, the same were provided, except mud. Eight randomly selected clams were stocked in each aquarium. Sampling was done weekly for 3 consecutive weeks starting 1 week after the initial analysis. (The experiment could not be extended for a longer time, because 3 weeks is the maximum period the clams can survive in seawater without substrate.) Two clams were randomly chosen from each aquarium and processed as in the initial analysis. Gill coloration was scored (very light brown = 2; light brown = 4; brown = 6; dark brown = 8; and blackish brown = 10) and correlated with its elemental sulfur content.

Temperature, salinity, pH, dissolved oxygen, water and organic matter content, and total sulfur content of the mud were taken during the initial and the weekly samplings.

Total Sulfur Analysis

For the total sulfur content analysis, sediment samples were air dried and ashed in an Automatic Muffle Furnace MFD-200N at 700 °C for 40 min. Total sulfur was extracted using hydrochloric acid, processed as in elemental sulfur and the absorbance measured at 440 nm (Beaton et al. 1968). Total sulfur was expressed in percentage. All analyses were conducted at the Centralized Analytical Laboratory of SEAFDEC/AQD. Elemental sulfur contents were compared: (1) among tissues; (2) with time; and (3) between treatments (with or without mud substrate). Analysis of variance (ANOVA) was used to determine significant differences between means of elemental sulfur content of tissues at different times and treatments ($\alpha = 0.05$) (SAS 1988) and Duncan's Multiple Range Test (DMRT) to isolate these differences (Gomez and Gomez 1984). Weekly means of sulfur content of clams reared with and without mud substrate were compared using *t*-test (SAS 1988). "Clams" in the text refer to *A. edentula*, unless otherwise stated as *A. antiquata*.

RESULTS

During sample collection, substrate temperature ranged from 27.0–30.0 °C (mean = 28.70); salinity 33.0–36.0 ppt (mean = 35.0); pH 5.15–6.55 (mean = 5.63); D.O. 0.2–1.0 ppm (mean = 0.38); water content 49.4–56.0% (mean = 53.4); organic matter 5.08–6.58% (mean = 6.24); and total sulfur content 1.3–2.2% (mean = 1.8). There was no rainfall during the entire collection period.

A. edentula were collected at depths ranging from 0.28–0.50 m from the surface. Its foot was observed to extend several times longer than the clam's shell length. On the other hand, *A. antiquata*, which were analyzed for comparison purposes, thrive at

shallower depths (surface–0.1 m). Its foot was hatchet-like and does not extend longer like the foot of *A. edentula*.

During laboratory culture of the clams, water temperature ranged from 27.0–28.5 °C (mean = 27.9); salinity 33.0–35.0 ppt (mean = 34.0); pH 7.6–8.3 (mean = 8.05); and D.O. 3.4–6.0 ppm (mean = 4.41). Total sulfur content of the substrate ranged from 0.3–0.7% (mean = 0.4); water content 49.0–66.8% (mean = 58.18), and organic matter content 5.15–6.58% (mean = 5.72).

The organic matter content of the newly collected substrate (6.24 ± 0.29%) was not significantly different from those stocked with clams (5.72 ± 0.57%) for 3 weeks. However, sulfur content of mangrove mud *in situ* was higher (1.8 ± 0.15%) than the one used as substrate in the experiment (0.4 ± 0.1%); the latter had no significant difference from initial to final readings.

In newly collected clams, the gills were deep brown to deep purple, thick and fleshy, as compared with the gills of individuals kept for 3 weeks in the aquaria, which were thin and filamentous. In the course of the 3-week sampling period, there was a slight fading of color in the gills. Correlation analysis showed no relationship between gill coloration and elemental sulfur content. However, gills of newly collected *A. antiquata* were reddish brown, thin, and filamentous.

There was not much change in the total wet weight of the clams. Newly collected clams were slightly lighter (36.01 ± 2.06 g) compared with clams weighed weeks later (36.84 ± 2.05 g) during laboratory experiment.

Elemental sulfur levels were significantly different (ANOVA, $P < 0.0001$) in the gills (247.64 ± 63.28 μmoles/g FW) compared with the mantle (0.84 ± 0.22 μmoles/g FW). Elemental sulfur was absent from the foot tissues (Fig. 2). Results also showed a significant decrease (ANOVA, $P < 0.01$) in elemental sulfur between the newly collected clams and those stocked in aquaria (both in mud and without mud substrate) and analyzed 1–3 weeks later (Fig 3a). Minimal sulfur was detected in the mantle only at the start of the experiment (Fig. 3b).

Results further showed that elemental sulfur in the gills of clams stocked in mud substrate were significantly higher than those stocked in seawater only for weeks 1 (*t*-test, $P < 0.05$) and 2 (*t*-test, $P < 0.05$). However, there was no significant difference between these two treatments in week 3 (Fig. 4). For the mantle, there was no significant difference between treatments. No elemental sulfur was detected in the mantle, gill, and foot tissues of *A. antiquata* (data not shown).

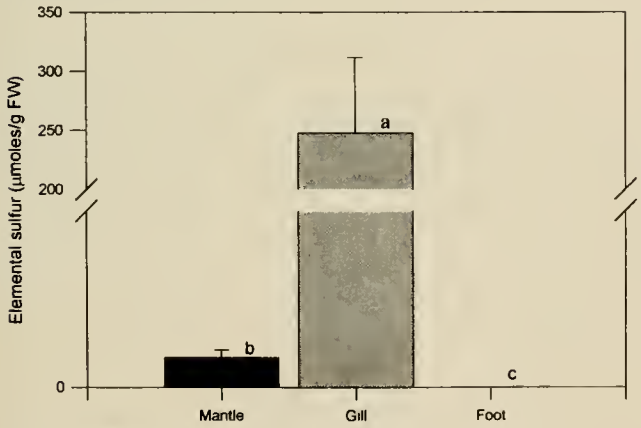

Figure 2. Means ± SE of elemental sulfur content of different tissues of *Anodontia edentula* (ANOVA, $P < 0.0001$).

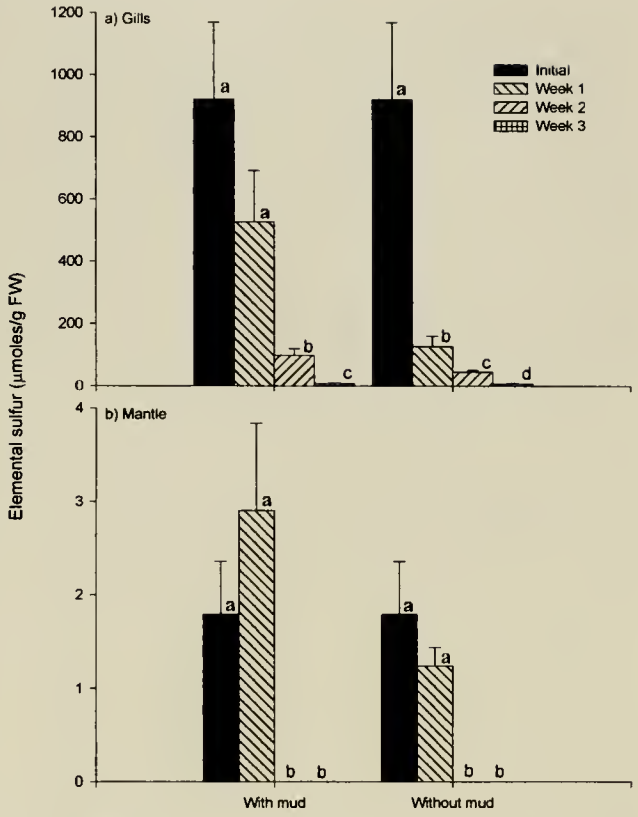

Figure 3. Means ± SE of elemental sulfur content of *Anodontia edentula* a) gills and b) mantle from initial to week 3 readings in clams stocked in mangrove mud substrate and in seawater only. Means with the same superscript are not significant. (ANOVA, $\alpha = 0.05$)

DISCUSSION AND CONCLUSION

The major natural sources of sulfur in the soil are organic matter and soil minerals. Most of these are bound, but over time, microorganisms can simplify them into soluble inorganic forms (Brady 1990). Hydrogen sulfide and other sulfides are produced during mineralization of organic sulfur (proteins and other organic combinations) under anaerobic conditions (Atlas 1995, Brady

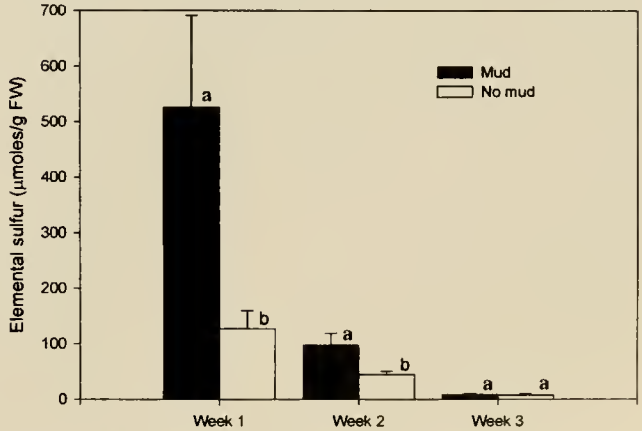

Figure 4. Means ± SE of elemental sulfur content of *Anodontia edentula* gills between treatments from week 1 (*t*-test, $P < 0.05$), week 2 (*t*-test, $P < 0.05$) week 3 (*t*-test, $P > 0.05$). Means with the same superscripts are not significant.

1990). These sulfide ions will undergo hydrolysis to form gaseous H_2S causing the rotten egg smell of swampy or marshy areas (Brady 1990) including mangroves where *A. edentula* thrives.

In a clam–bacteria symbiotic relationship, the clam's anatomy and its location in the substrate/mud play very vital roles. According to Distel and Felbeck (1987), clams should be strategically situated in an interface between a sulfide-generating zone (anoxic) and water with sufficient oxygen (oxic). The foot of *A. edentula*, which can extend up to several times longer than its length, gives it the capacity to construct a ventilation burrow (Reid and Brand 1986) and allows it to draw water from the surface (Dando et al. 1985). The tip of the foot is specialized for the construction of this inhalant tube. It is provided for with glands that lay down mucus for the building of the tube (Allen 1958). The clam gains access to oxygenated water through this inhalant tube. Its location (0.28–0.50 m deep in mangrove mud) may allow it direct access to sulfide. In cases where sulfide is limiting, Childress et al. (1991) have proved that the foot can dig deeper and is responsible for the uptake of sulfide from deeper parts of the substrate. Sulfide is taken up across the foot of the clam and into the blood that transports it to the gills for use of the bacterial symbiont (Ruppert and Barnes 1994).

Sulfide is a highly reduced energy molecule and a variety of biological systems have evolved to oxidize sulfide in orderly enzyme-regulated steps to harness the energy and avoid poisoning (Bagarinao 1992). In a clam–bacteria symbiosis, bacteria are assumed to provide the mollusk with chemosynthetically fixed carbon dioxide via aerobic oxidation of sulfide (Vetter 1985). Oxidation of sulfides and other reduced sulfur compounds provides energy to the bacteria to fix carbon dioxide into organic compounds that become available to the host clam (Distel and Felbeck 1987, Kelly and Harrison 1989). The Calvin cycle is the main metabolic pathway used by the bacteria to convert carbon dioxide to organic carbohydrates powered by the energy (ATP) generated from the oxidation of sulfides (Atlas 1995). In the process of oxidation, sulfide is converted into elemental sulfur and stored for future use (Childress and Mickel 1982, Vetter 1985). Using EDX microanalysis, Reid and Brand (1986) found sulfur as the dominant elemental inclusion of bacteria in a lucinid clam *Parvilucina tenuisculpta*.

In this study, elemental sulfur was present in highly significant amounts in the gills (max: 1907.20 μmoles/g FW from a newly collected clam; min: 1.38 μmoles/g FW from a clam reared without substrate and analyzed at week 3) (Fig 3) and based on the compilation of Somero et al. (1989), these values are within the range of elemental sulfur content (0.22–2593.8 μmoles/g FW) for different species of bivalves containing symbiotic bacteria. The very wide difference between the maximum and minimum values may be attributable to the mobilization of elemental sulfur by the bacteria in the absence of external sulfide (Vetter 1985) in aquaria not provided with mud substrate. This is clearly exhibited by the significant decrease in elemental sulfur content of the gills from the initial sampling to week 3 (Figs. 3a, 4). The initial samples showed to have significantly higher elemental sulfur content than those stocked in mud and those in seawater (Fig. 3a). Furthermore, clams stocked in mud have significantly higher sulfur content at weeks 1 and 2 than those stocked in seawater only. However, at week 3, there was no significant difference between the two treatments. In the first 2 weeks, bacteria in clams maintained in mud may have utilized all available sulfide in the mud; whereas, those stocked in seawater only may have depended on their elemental sulfur reserves. Moreover, around week 3, the clams stocked in mud may have also utilized their sulfur reserves because of inadequate sulfide supply in the mud. In the absence of external sulfide, elemental sulfur represents a novel inorganic energy reserve for the animal–bacterial symbiosis (Vetter 1985), which, according to Powell and Somero (1985), is the most reduced nontoxic inorganic form of sulfur.

If sulfur globules are confined in the periplasmic space of bacteria (Vetter 1985) then *A. edentula* must have these endosymbiotic bacteria in their gills to store sulfur.

The presence of elemental sulfur in very minimal amounts in the mantle may be attributable to the connection that links the mantle and the gill, which may have served as a passage to some bacteria containing elemental sulfur globules. There is an insertion and fusion of the posterodorsal margins of the gills with the muscular posterior mantle edge (Reid and Brand 1986). However, this may also be attributable to contamination during dissection.

The deep brown to deep purple, thick and fleshy gills of *A. edentula* were also observed by Felbeck et al. (1981) in bivalves collected from sulfide-rich habitats containing sulfide oxidation enzymes in contrast with the small, light-colored gills of those lacking these enzymes. According to Distel and Felbeck (1987), the dark coloration and thickness of the gills are attributable to the presence of a thick layer of subfilamentar tissue perforated by regular arrays of bacteriocyte channels formed by bacteriocyte cylinders containing the bacterial symbionts.

The very slight, nonsignificant increase in wet weight of cultured clams (3 weeks) compared to the newly collected ones used for the initial analysis may be caused by the water trapped inside the clams' body cavity. Newly collected clams were not weighed immediately in the field but were transported to the laboratory and weighed 8 h after collection. Water trapped inside the body cavity was exuded. During laboratory experiment, clams were weighed immediately upon harvest and so water trapped inside the body cavity was released only during dissection and has, therefore, added to the weight of the individual.

The absence of elemental sulfur in *A. antiquata* simply shows that only lucinid clams harboring endosymbiontic bacteria in their gills are capable of sulfur-oxidizing metabolism and have the capacity to store these sulfur globules.

The results of the experiment, therefore, support the presence of endosymbiotic bacteria in *A. edentula* as exhibited by the presence in significantly higher amounts of elemental sulfur in the gills.

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I am grateful to the Philippine Council for Aquatic and Marine Research and Development (PCAMRD) of the Department of Science and Technology (DOST) for the study grant; SEAFDEC/AQD for the use of laboratory facilities and field equipment; J. D. Taylor of the Natural History Museum (London) for the identification of *A. edentula;* J. H. Primavera for the invaluable help throughout the study; J. A. Ingles, A. del Norte-Campos, N. B. Armada, L. V. Laureta, T. U. Bagarinao, E. de Jesus, and N. Golez for carefully reviewing the manuscript and for their suggestions and recommendations; F. Harder, A. Asutilla, P. Bantilan, A. Andrada, and M. B. Lavalle for the laboratory assistance; V. Balinas and D. Meciano for the statistical analyses; E. Ledesma for the figure; and the Bata-anon couple for their expertise in clam collection.

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EVALUATION OF TAG TYPES AND ADHESIVES FOR MARKING FRESHWATER MUSSELS (MOLLUSCA: UNIONIDAE)

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ABSTRACT Prior to initiating a long-term tagging program on freshwater mussels, we evaluated three varieties of tags (Northwest Marine Technology Visual Implant Tag, Floy Fingerling Tag, and Hallprint Shellfish Tag) two types of adhesives (3M two-part epoxy and Krazy Glue cyanoacrylate), and four bonding times before immersion in water (2, 5, 10, and 15 min). Tags were applied to empty shells for two phases of testing. First, legibility was visually scored for each of the immersion times. The two-part epoxy became cloudy at immersion times < 15 min, was easily abraded after curing, and was, therefore, eliminated from further testing. The visual implant tag also was eliminated from further testing because the printing dissolved in the cyanoacrylate. In the second phase, the fingerling tag and the shellfish tag bonded with cyanoacrylate were tested for durability and retention under natural conditions in a shallow stream and under highly abrasive conditions in a standard gem tumbler containing coarse metal shavings. Tag losses after 16 wk in the instream test were 5.3% for the shellfish tag and 2.6% for the fingerling tag. These tests revealed no loss of legibility after the removal of material that accumulated on the tag surface. There was no appreciable wear of either tag type after 1 wk in the gem tumbler. Flexible polyethylene shellfish tags were chosen for field application because they are thinner and available with a larger number of individual codes than fingerling tags. Tags bonded to the shells of live mussels with cyanoacrylate can be immersed in water in as little as 2 min after application without affecting retention. This method was used to double-tag 1,372 mussels at a monitoring site on the Cacapon River in West Virginia. After 2 y, the total tag loss in 325 recovered mussels was 0.46%.

KEY WORDS: Freshwater mussels, tag, adhesive, tag retention

INTRODUCTION

External identification of individual freshwater mussels is highly desirable for tracking passive and active movements, growth analyses, population studies, and laboratory experiments. The marking of freshwater mussels is particularly problematic because these are long-lived species, and programs to collect population information could potentially last for many years, requiring excellent long-term legibility and retention. Additionally, marks on freshwater mussels are subjected to abrasion from animal movement through mineral substratum and water-borne materials in fast-flowing water. Any marking method must have minimal effects on the survival, growth, and behavior of the tagged animal. The ideal marks should be easy to apply, inexpensive, and readily available, especially when needed for large-scale marking programs. In addition to high legibility, the tags should be small and of subdued colors to avoid the potential for increased susceptibility to predation in field studies (although bright colors for easy visibility in laboratory studies may be desirable) and should be commercially available with a large number of codes.

Freshwater mussels have been individually marked by scratching numerals (Isley 1914, Couilliard et al. 1995), drilling codes in the periostracum (Thoma et al. 1959), attaching pendant tags with wire passed through a hole in the shell (Isley 1914), and attaching plastic labels with adhesive (Neves and Moyer 1988). Sequentially numbered plastic tags have been obtained from commercial vendors (Neves and Moyer 1988) or created with Dymo label makers (Balfour and Smock 1995). Adhesives used for plastic tags include dental cement (Downing and Downing 1993), wet surface repair putty (Balfour and Smock 1995), and cyanoacrylate (Neves and Moyer 1988). Cyanoacrylate also has been widely used to attach plastic tags to a variety of marine shellfish, including abalone (McShane 1989), scallops (Heald 1978, Williams and Dredge 1981, Gwyther 1989), and limpets (Treble et al. 1993). Neves and Moyer (1988) used this combination for freshwater mussels and found no apparent effects on growth or survival up to 4 y later. While long-term tag retention and legibility have been documented for freshwater mussels, they have not been quantitatively evaluated.

Because we desired to establish retention and legibility information before initiating long-term tagging programs, we conducted several short-term tests to select tags and adhesives. We elected not to scratch codes into the periostracum for fear that this action could cause increased shell erosion and the high-contrast marks could make the animals more visible to predators. Two-year retention and legibility results from a field monitoring study, initiated during the summer of 1996, also are presented here.

MATERIALS AND METHODS

The fingerling tag and the visible implant tag (so named for its intended use in clear tissue on fish) were initially selected and were used in adhesive immersion and legibility tests (Table 1). Shellfish tags were acquired after the adhesive tests were complete and were used in all subsequent evaluations. Shellfish tags are similar in size to the fingerling tags but are significantly thinner and carry larger characters and a greater number of characters. Both the fingerling tag and shellfish tag were previously used for marking freshwater mussels (R. Neves pers. comm.).

We selected adhesives that were readily available at reasonable cost, easily dispensed, fast curing, and known to be durable while immersed in water. A fast-curing adhesive was particularly desirable to minimize emersion time, thus reducing stress to the mussels

Reference to trade names does not constitute U.S. Government endorsement of commercial products.

TABLE 1.

Tags used in evaluations.

Characteristics	Fingerling Tag	Visible Implant Tag	Shellfish Tag
Manufacturer's model no.	FTF-69	None	FPN
Manufacturer	Floy Tag and Manufacturing, Inc., Seattle, WA	Northwest Marine Technology, Shaw Island, WA	Hallprint Pty. Ltd., Holden Hill, SA, Australia
Tag size (mm)	3.2 × 4.8	1.0 × 2.5	4 × 8
Tag thickness (mm)	0.65	0.09	0.15
No. of characters	3 numeric	1 alpha + 2 numeric	1 alpha + 3 numeric
Character height (mm)	1.3	0.7	1.8

and providing a more efficient tagging process. Because tags will be exposed to highly abrasive conditions as animals move through the mineral substrate, our initial assumption was that it would be necessary to cover the tags with clear adhesive to protect the printed codes, especially in the case of the visible implant tags, which are very small and delicate. A two-part epoxy (DP-100, 3M, St. Paul, MN) and a cyanoacrylate (Krazy Glue, Borden, Inc., Columbus, OH) fit all of these considerations, and were selected for the initial round of tests.

The tag and adhesive combinations were tested in a two-phase process. First, we tested the ability of the adhesive to form a secure bond and to remain clear when immersed in fresh water shortly after application. Second, we evaluated the durability of the selected adhesive and tag types when exposed to natural stream conditions and a highly abrasive environment. Tags were applied to empty shells collected from local streams. To prevent exfoliation of the periostracum due to drying, all shells were stored in water before and after tagging. Unsatisfactory adhesives and tags were eliminated at each step. The tag and adhesive combination that was found to perform best in these initial tests then was used to double-tag live mussels for monitoring in a multiyear field study. Double-tagging assumes that both tags are retained independently and can be used to estimate tag loss (Arnason and Mills 1981).

Evaluation of Adhesives

Adhesives were evaluated initially by attaching tags to the exterior surfaces of empty shells and allowing them to air dry for 2, 5, 10, and 15 min prior to immersion in water. A maximum duration of 15 min was chosen to minimize the potential stress to live animals and to maintain efficient tagging rates in field applications. Ten replicates of each treatment (tag type, adhesive, time to immersion) were prepared. All tags were completely covered with the adhesive. Tagged shells were stored in 1-L containers filled with spring water. Weekly observations were conducted through 4 wk to evaluate legibility and the retention of the tags. Shells were removed from the water to conduct the observations, then immediately were replaced. Legibility was scored by a single observer (DPL) as easy to read (i.e., it could be read immediately), difficult to read (i.e., it required close scrutiny or scraping of encrusting glue), barely legible (i.e., despite the aforementioned efforts, an 'educated guess' was still necessary to make out the numbers), or illegible. Numeric scores of 3 through 0 were used to calculate mean legibility scores for each treatment.

Evaluation of Tags

Based on the results of the initial tests, only cyanoacrylate was used for further testing. In addition, due to legibility problems to be discussed later, the visible implant tags were replaced with shellfish tags for subsequent evaluations (Table 1).

Instream Immersion Test

As in the adhesive test, shells were immersed 2, 5, 10, and 15 min after the application of tags. After remaining in water for 1 wk, one shell from each treatment was imbedded in a natural position in a block of concrete. Ten replicate blocks were prepared. Blocks were placed perpendicular to the current in a section of Hopewell Run, a second-order stream in Jefferson County, WV, with fairly homogeneous water depth and velocity. The blocks were randomly rearranged weekly to ensure equal exposure to variations in flow conditions. Tags were examined after 16 wk to evaluate legibility and retention.

Tumbler Test

A minimum of four examples of both tag types were applied to mussel shells using cyanoacrylate, which was allowed to cure for 2–3 min then was immersed in water. To fully test the durability of the tag material and printing, care was taken to avoid placing adhesive on the surface of the tags. After remaining in water for approximately 24 h, individual shells were placed in a rock tumbler (Natural Science Industries, Far Rockaway, NY) containing 38 g of coarse metal shavings. The tumbler was operated for 1 wk with periodic examinations.

Field Tests

Shellfish tags were attached to live mussels with cyanoacrylate and were monitored at a site on the Cacapon River in West Virginia. The site was 200 m long and was divided into 10 20-m long sections. Each section was searched for 30 min using viewing buckets, and all mussels encountered were removed from the substrate for identification, measurement, and tagging, then they were returned to the substrate in the same section where they were found. Each animal was double-tagged with one tag placed on the posterior slope of each valve. The area of tag application was cleaned with a medium grit sandpaper or scrub pad, then was rinsed and blotted dry. A small amount of cyanoacrylate was applied to the shell (i.e., a volume sufficient to completely attach the tag with minimal excess glue), then, using forceps, the tag was placed on the adhesive and pressed gently.

The adhesive was allowed to air dry for at least 2 min before returning the animal to the water. The initial survey and tagging were conducted in July 1996, with subsequent surveys conducted in January, April, June, and October 1997, and in June 1998. Additional animals were tagged at each interval. Water temperature and turbidity were measured during each survey.

RESULTS

Evaluation of Adhesives

The characters on the fingerling tag were normally readable without magnification. The characters on the visible implant tags, however, are approximately 0.7 mm in height and were most easily read with the aid of a magnifying lens. Regardless of tag type (Fig. 1), the epoxy did not perform well, becoming cloudy when immersed for times < 15 min. Legibility in the 15-min group was impaired by the roughness of the surface of the adhesive. Additionally, the epoxy remained soft and was easily abraded during examination.

The printing on the visible implant tags dissolved in cyanoacrylate, resulting in complete loss of legibility in all tags in this treatment. Cyanoacrylate provided good visibility with the fingerling tags regardless of time to immersion, although minor surface rippling was noted in the groups immersed 2 and 5 min after application.

Evaluation of Tags

Instream Immersion Test

The adhesive covering the tags acted as a substrate for the attachment of silt and periphyton, somewhat impairing visibility. It was frequently necessary to scrape the surface of the glue to remove attached material. In some cases, it was necessary to remove the glue from the surface of the tag by gently scraping with a knife. There was no loss of legibility after surface materials had been removed. Tag losses for shellfish tags and fingerling tags were 5.3% and 2.6%, respectively, with no apparent relationship to the four drying times prior to immersion.

Tumbler Test

Neither tag type showed significant wear when compared to new tags, and no tags were dislodged during the tumbling process. As an extreme test, a shell with a shellfish tag was tumbled for an additional 3 wk with metal shavings, plus 4 wk with up to 100 g of sand in the chamber, and still did not show any appreciable wear.

Field Tests

Between June 1996 and October 1997, 1,372 mussels were tagged at the Cacapon River site. Species consisted of predominantly *Elliptio complanata* (Lightfoot, 1786) (85.6%), *Elliptio fisheriana* (Lea, 1838) (7.7%), and *Lampsilis cariosa* (Say, 1817) (5.9%) and a few individuals of *Alasmidonta varicosa* (Lamarck, 1819), *Lasmigona subviridis* (Conrad, 1835), and *Strophitus undulatus* (Say, 1817) (total < 0.9%). During periodic surveys through June 1998, 325 tagged mussels were recaptured. The time at large for recaptures ranged from 69 to 722 days. Survey temperatures ranged from < 0 to 24°C, and turbidity ranged from 1.4 to 14.3 NTUs.

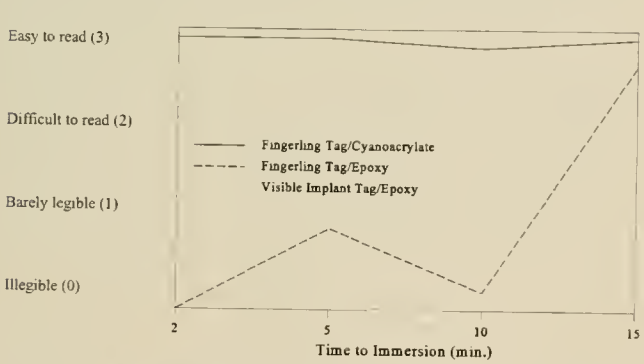

Figure. 1. Mean legibility of tag and adhesive combinations (numeric score) after immersion in water.

Only three tags were lost (0.46% of all tags recovered); two were missing when recaptured after 70 and 378 day *in situ*, and one was rubbed off while removing an attached caddisfly case after 447 day *in situ*. Loss of legibility was observed only once, after 69 days *in situ* (0.15%) and may be attributed to a printing error that was not recorded at the time of tagging. Additionally, six tags (0.92%) were reported to be loose when examined 69–343 days after tagging. Typically, one end of the tag was not well-adhered to the shell surface.

DISCUSSION

The results of this study indicate that the shellfish tag adhered with cyanoacrylate provides a good long-term marking method for freshwater mussels. While any tag loss is undesirable, the minimal losses observed in the field tests would not seriously affect population estimates based on these surveys. Conducting tests in the laboratory eliminated the need for trial and error exercises in the field and allowed the establishment of protocols before project initiation. This testing process eliminated an adhesive with undesirable characteristics, avoiding tag losses and illegibility that may have impacted long-term studies.

Although the two-part epoxy formed a clear, hard bond after a 15-min drying time, we felt that this was too long for safe handling of the mussels and would adversely affect the efficiency of field survey operations. Cyanoacrylate bonded quickly and was preferable because it did not require mixing. We also found that it was not necessary to cover the tag with adhesive, as the tag surface and printing are highly durable. Tumbler tests indicated that the printing on both tag types is of sufficient durability that complete coverage of the tag with adhesive is unnecessary. Clear adhesive, however, is still desirable in the event that glue gets on the tag surface.

Tag retention was greater in the field tests than in the instream immersion tests. Because old shells collected in previous surveys were used, higher losses may have been associated with a decrease in the integrity of the shell surface, even though the shells had been stored in water prior to tagging. In addition, tags on empty shells were more exposed to flowing water than those on live animals that could burrow into the substrate. Although water conditions were measured in both the stream immersion test and field tests, they were measured only at the observation intervals and may not reflect the full range of conditions. The evaluation of adhesive durability under different water conditions (e.g., temperature range, hardness, and alkalinity) should be undertaken in the future.

For our freshwater mussel surveys, shellfish tags are preferred over the fingerling tags for several reasons. Primarily, they are much thinner, reducing the opportunity for objects to snag on the tag edge. Treble et al. (1993) suspected that losses of tags were caused by abrasion and snagging as limpets moved through narrow crevices. Shellfish tags are also available in a wider range of colors, including subdued natural colors that may reduce visibility to predators (e.g., gray and beige). In addition, they use a combination of letters and numerals, which provides a large number of

individual codes for each tag color. Finally, the shellfish tags come on a roll, rather than loose, making them easier to handle in the field.

While costly in terms of the number of tags and the time to mark, the use of double tags will benefit large-scale, long-term tagging programs. It will allow a continuous evaluation of tag losses, the rate of which may change over time (Treble et al. 1993). Establishing rates of tag loss will provide correction factors for population estimates based on recapture of tagged individuals (Seber 1982, p.94). Because entire tags can become obscured by algae, insect cases, and other materials, the use of two tags also may increase the possibility that a tagged animal is recognized. Although these tests were physically rigorous, it is uncertain how long the adhesive will be effective. Differences in tag retention among species due to morphology (e.g., periostracum texture or animal size) and behavior (e.g., substrate preference or burrowing depth) are also unknown. Long-term monitoring and reporting to the research community will aid in the development of future tagging programs.

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BACTERIAL PATHOGEN CONTAGION STUDIES AMONG FRESHWATER BIVALVES AND SALMONID FISHES

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ABSTRACT A part of the conservation efforts of native freshwater bivalves is a relocation program whereby animals are collected and moved to a safe refuge for maintenance and propagation. With the rearing of two different hosts, mussels and fish, on the same facility there is a question of the possibility for contagion of pathogens. The studies presented here are part of a continuing effort to address the concerns of contagion. Freshwater bivalves collected throughout the 1997 season were cultured for fish pathogens. Counts of total bacteria on cytophaga medium ranged between 1.07×10^5 and 4.99×10^5 cfu/g of mussel soft tissues. The predominate groups of bacteria were motile *Aeromonas* spp. and *Pseudomonas* spp., both of which include members that are opportunistic pathogens to salmonid fishes. No primary fish pathogens were cultured; however, cells with correct morphology for *Renibacterium salmoninarum*, cause of bacterial kidney disease, were detected from mussel soft tissues in all six trials using the direct fluorescent antibody test. Groups of mussels were subjected to 24-h waterborne challenges using bacteria cultured from healthy fish; no mortality occurred to any of the animals. Another group exposed to the fish pathogen *Aeromonas salmonicida* also showed no effects of the challenge; however, susceptible fish became infected and died after the fish were added to cohabit with this group of mussels.

KEY WORDS: Freshwater bivalves, salmonid, pathogen, disease, contagion, bacteria, cohabitation

INTRODUCTION

Over 70% of the approximately 300 species and subspecies of freshwater bivalves (*Unionidae*) native to North America are categorized as endangered, threatened, or of special concern (Williams et al. 1993). Bivalves are particularly susceptible to impacts from environmental changes, not only because they are sessile, filter-feeding animals, and adverse effects placed on their intermediate fish hosts that are necessary for glochidia development also may indirectly affect them.

A number of factors have been documented as contributors to the decline in numbers of freshwater bivalves. Human disturbance in the terrestrial environment, such as agriculture and development, result in siltation that may impair growth and respiration and lead to suffocation (Ellis 1936, Kat 1982). Also, habitat alteration from dredging or dam construction can result in changes in flow or temperature and also affect movement of the intermediate fish host (Fuller 1974; Keller and Zam 1990). In recent years, the zebra mussel (*Dreissena polymorpha*) has become a major threat to native bivalve populations in large river systems (Herbert et al. 1991; Nalepa 1994). Zebra mussels are able to proliferate and compete very favorably against native animals (Gillis and Mackie 1994). Their high spatial tolerance allows them to colonize in great numbers, leading to mortality of native bivalves by impeding feeding and respiration (Griffiths et al. 1991, Haag et al. 1993, Leach 1993, Mackie 1991). In 1996, densities of zebra mussels in the lower Ohio River (near river mile 814) exceeded 14,000 per square meter, and mortality to the native populations exceeded 30% (P. Morrison, Ohio River Islands National Wildlife Refuge, U.S. Fish and Wildlife Service, pers. comm.).

In the mid 1990s, the U.S. Fish and Wildlife Service (USFWS) along with other federal, state, and private partners initiated conservation efforts. One such program was to isolate native animals from impending zebra mussel infestation from selected large rivers. Under this program, individuals are collected and relocated to safe refugia for maintenance and propagation with the hope of future successful reintroduction. These refugia, which are free of zebra mussels, include salmonid fish-rearing hatcheries, which may culture such species as rainbow (*Oncorhynchus mykiss*) and brook (*Salvelinus fontinalis*) trout. With the rearing of these two hosts (mussels and fish) on the same facility, there exists the possibility for contagion of pathogens either by shared water or via contaminated equipment such as boots, nets, and buckets. The question of contagion is particularly relevant, because one of the two hosts (freshwater mussels) is originating from a natural environment and might be exposed to pathogens of both mussels and fish that could be introduced to a hatchery along with their relocation. All of the recognized primary bacterial pathogens of salmonids involve, to varying degrees of significance, horizontal transmission, and, hence, there is a potential to develop disease in fish that might be exposed to pathogens via contamination or by some vector. It can be speculated that bivalves may serve as a pathogen vector. On the other hand, it is not known if certain microbial flora of healthy resident fish might pose a disease threat to mussels once they are exposed to the pathogen, and they are reared in intensive culture situations. There are no single pathogens described that produce disease in both salmonid fishes and freshwater bivalves. There is a wealth of knowledge on diseases to salmonids, but there are few reports of diseases and epizootics that occur in native freshwater bivalves. However, it has been demonstrated that freshwater mussels may serve as a vector for fish pathogens, because *Flavobacterium columnare*, the cause of columnaris disease, was isolated from a single *Amblema plicata* that was collected from the Ohio river (Starliper et al. 1998).

Presented here are studies that are part of a continuing effort to evaluate the potential for contagion of bacterial pathogens. We report on efforts to isolate salmonid bacterial pathogens from freshwater bivalves that were collected from their natural environment (without quarantine), and to produce disease or mortality

experimentally in bivalves using the representative bacterial flora from healthy fish and two fish bacterial pathogens.

MATERIALS AND METHODS

Freshwater Bivalves and Fish

All bivalves were collected by brailing between August and November 1997, and this duration was representative of that year's sampling season. Duration of sampling seasons varies from one year to another, depending upon water temperature and when animals emerge from the river bottom. The collection site was between river miles 175 and 177 at Muskingum Island of the Ohio River, which is near Boaz, Wood County, West Virginia. This region of the river is not easily accessible by land, and the only public use is pleasure boat traffic. The site supports 28 species of freshwater mussels, including two federally listed species (*Lampsillis abrupta* and *Cyprogenia stegaria*). The island and its underwater acreage, including the mussel beds, are protected from development, because it is within the Ohio River Islands National Wildlife Refuge. No harvesting or collecting of mussels is allowed on the refuge except for scientific or management purposes.

Bivalves used to evaluate the presence of fish pathogenic bacteria were collected at 2 to 3 week intervals during this season. Twenty animals were collected on each of six dates (trials 1–6, respectively), except for trial 6, when only eight were collected because of low water temperature. Upon collection, animals were kept cool and moist and shipped overnight by commercial carrier to our laboratory for bacteriological analysis the following day. They were not placed in other water in the interim between collection and analysis. Species and physical data for the bivalves represented in this study are presented in Table 1. Also, the distribution data for *Amblema plicata*, which was the species most frequently collected during the season, are given in Table 2. Ohio River surface water temperatures (°C) were recorded at the time and collection location for each trial.

Two hundred animals, representing six species, were used as subjects for bacterial challenges with flora isolated from healthy fish: *A. plicata* (123), *Quadrula metanevra* (33), *Q. quadrula* (21), *Obliquaria reflexa* (10), *Q. pustulosa* (9), and *Pleurobema cordatum* (4). Physical data of the animals used in the challenge studies are not presented; however, mean values were very similar to those animals used for fish pathogen isolation, which are presented in Tables 1 and 2. These animals were quarantined for 30 days at the Ohio River Islands National Wildlife Refuge (Parkersburg, WV) to eliminate any zebra mussels (Gatenby et al. 1998). They were then transported to the Leetown Science Center, where they were acclimated to (over 2–3 hours) and maintained in pathogen-free spring water (12 °C) delivered via a flow through system.

The fish added to the tanks to cohabit with mussels following their (mussels) challenge were the Nashua strain of brook trout (*Salvelinus fontinalis*), about 50 g each, and the Shasta strain of rainbow trout (*Oncorhynchus mykiss*), also about 50 g each. The fish were certified as fish pathogen-free through biyearly fish health inspections by the U.S. Fish and Wildlife Service's Fish Health Unit, Lamar, Pennsylvania. The fish were maintained in the same water as described for the mussels; all *in vivo* studies were also done using the same water source.

Collection and Processing of Tissues from Bivalves

Tissues were collected and prepared using a procedure similar to that developed by Starliper et al. (1998). The external shell surface of each animal was cleaned by brushing with 200-ppm chlorine, rinsed in deionized water, and allowed to dry. An oyster knife was used to pry open the shell valves, and the adductor muscles were cut. All soft tissues were excised from the shells and were separated into two samples. One consisted primarily of digestive tract tissues, denoted "gut" (e.g., stomach, intestine), while all remaining soft tissues, denoted "OT" or other tissues, (e.g., mantle, gill, foot, lymph) comprised the second. The gut sample was removed first, and effort was made not to contaminate the remaining tissues with bacterial contents from the gut. Soft tissue samples were placed in preweighed, sterile stomacher bags. The bags with tissues were weighed, and the difference of the two weights was the weight of the tissue sample.

Isolation and Growth of Bacteria

For bacterial isolation, the tissue samples were diluted in sterile 0.1% peptone- 0.05% yeast extract (PEP-YE). To each sample, a volume (mL) of PEP-YE equal to the tissue weight (g) was added yielding a 1:2 dilution of tissues. This was homogenized for 120 seconds using a Model 80 stomacher (Seward Medical, London SE1 1PP, UK). A portion of the supernatant from each homogenate was transferred to a sterile tube for ease of handling and three serial tenfold dilutions were prepared from this, also in PEP-YE. Four drops (0.025 mL each) from all dilutions were applied to the surface of each bacteriological medium. After the drops had been adsorbed, plates were incubated at the appropriate temperature and duration specified in the appropriate reference materials (see Media employed). Following incubation, colonies were enumerated by counting the lowest dilution with single colonies, and this was converted to a standard colony forming units per g of tissue (cfu/g). For the selective and/or differential media used for specific

TABLE 1.

Mean values for physical data on freshwater bivalves from the Ohio River assayed for presence of bacterial fish pathogens. Animals were collected at six different times (trials 1–6) during the 1997 collection season.

Species	No.	Length (mm)	Width (mm)	Weight (g)	% Soft Tissue[a]	Gut/OT %[b]
Amblema plicata	88	100.9	74.4	238.5	12.5	61/39
Quadrula quadrula	7	74.1	58.4	123.0	10.5	62/38
Obliquaria reflexa	5	58.8	46.8	75.2	11.2	64/36
Pleurobema cordatum	3	77.0	65.3	142.9	11.7	57/43
Quadrula metanevra	3	67.3	57.0	107.6	12.0	61/39
Quadrula pustulosa	1	57.0	54.0	63.1	15.2	53/47
Ellipsaria lineolata	1	45.0	34.0	25.7	10.9	56/44

[a] % Soft tissue = the percentage of the total weight that is comprised of soft tissue.
[b] Gut/OT % = the percentage of the total weight of soft tissue in gut and OT samples.

TABLE 2.

Mean values of physical data for *Amblema plicata* collected during the 1997 sampling season.

Trial	No. Animals	Length (mm)	Width (mm)	Weight (g)	Soft Tissue (%)[a]	Gut/OT Ratio[b]
1	13	105.8	76.9	269.9	11.9	59/41
2	18	102.4	76.5	247.6	11.6	59/41
3	18	101.9	76.9	252.4	12.1	62/38
4	20	87.9	64.3	153.7	12.7	61/39
5	17	108.6	78.7	283.2	13.9	64/36
6	2	112.5	80.5	294.7	13.1	63/37

[a] Percentage of the total weight of the bivalves that were soft tissues used for isolation of bacteria.
[b] Percentage of the total soft tissues used for: Gut (/) and OT samples for isolation of bacteria.

isolation of pathogens, suspect colonies that were picked to fresh media had their identity confirmed as to that particular pathogen, or not. Biochemical tests employed for bacterial characterizations were described in the appropriate reference papers or with standard biochemical characterization (Koneman et al. 1988; MacFaddin 1980). The sets of 1:2 dilutions were used to prepare smears on microscopic slides for detection of *Renibacterium salmoninarum* cells using the direct fluorescent antibody test (FAT; Bullock et al. 1980) and commercially available FITC-conjugated antiserum prepared in goats to the whole cells (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD). One hundred microscopic fields of view were observed from each stained homogenate at 1,000× magnification using a Reichert Diastar Model 420 microscope with a halogen light source (Cambridge Instruments Inc., Buffalo, NY). An *R. salmoninarum*-positive kidney tissue from a diseased salmon served as a control slide for reference to the correct cell morphology.

Isolation of *A. salmonicida* from fish that died as a result of cohabitation with *A. plicata* that were previously challenged with the bacterium was done using a sterile inoculation loop to collect kidney tissue, and this was used to inoculate primary isolation plates. The medium for isolation of *A. salmonicida* was tryptic soy agar (TSA; Difco Laboratories, Detroit, MI) supplemented with 0.01 % coomassie brilliant blue (CBB; Cipriano and Bertolini 1988). The identities of suspect, blue colonies were confirmed as *A. salmonicida* with standard biochemical characterization tests.

Bacteria used for the waterborne challenges of bivalves were isolated from rainbow trout at the White Sulphur Springs National Fish Hatchery, White Sulphur Springs, WV. This facility was selected, because mussels are currently being held there, and the resident populations of fish are healthy and well maintained; therefore, they would be representative hosts of normal flora bacteria that relocated mussels encounter, either via the fish as a source or from their common water supply. Mucus and kidneys from 100 randomly selected rainbow trout were used to inoculate BHIA and CYTO plate media (described in Media employed). The resulting bacterial growth on the primary isolation media was observed, and the most frequent colony types, on the basis of morphology, were noted. Subcultures of the predominate bacterial types were established by transferring representative single colonies onto fresh plates. These isolates were used to challenge the groups of freshwater bivalves.

Media Employed

Thirteen media were employed to isolate bacteria, including fish pathogens from mussels. These media are routinely used by fish health personnel to culture environmental bacteria commonly found on fish or in aquatic environments and for isolation of specific pathogens. Two were used to determine total bacterial counts, brain heart infusion agar (BHIA; Difco Laboratories, Detroit, MI) and *Cytophaga* agar (CYTO), a medium of reduced nutrient concentration (Anacker and Ordal 1959); plates were incubated at 22 °C for 48 hours. An additional set of CYTO plates were incubated at 15 °C for culture of *Flavobacterium psychrophilum*, cause of bacterial coldwater disease of salmonid fishes (Bernardet et al. 1996). The cooler incubation temperature is favorable for growth of *F. psychrophilum*, and it also retards the growth of other, environmental bacteria that facilitate isolation. Three media were used for Gram-positive bacteria. Rogosa SL (ROGO; Difco Laboratories, Detroit, MI) was used for *Lactobacillus* and related genera. Azide blood agar base (ABA; Difco Laboratories, Detroit, MI) containing 5% sheep erythrocytes (Bio-Whittaker, Walkersville, MD) was used for other species, including *Streptococcus* spp. and *Staphylococcus* spp. Incubation of ROGO and ABA plates was at 28 °C for 3 days. A third, the selective medium (SKDM) described by Austin et al. (1983), was used for isolation of *R. salmoninarum*, cause of bacterial kidney disease (Bullock and Herman 1988). To enhance growth of this bacterium, the sterilized SKDM was cooled and supplemented with 1% filter sterilized culture metabolite (Evelyn et al. 1990) before pouring plates. Following incubation at 15 °C for up to 4 weeks, suspect *R. salmoninarum* colonies (Sanders and Fryer 1980) were suspended in 0.5 mL PEP-YE; 100 µl of this was placed on a microscopic slide and air dried. The dried smears were subjected to the direct fluorescent antibody test, as previously described. Other media used were for isolation and enumeration of Gram-negative fish pathogenic bacteria and unless indicated, were incubated at 28 °C for 2 to 3 days. Two were for isolation of *Yersinia ruckeri*, cause of enteric redmouth disease. They were the differential medium described by Waltman and Shotts (SW; 1984) and the differential and selective medium of Rodgers (ROD; 1992). Suspect colonies were transferred to TSA. Two media were for *Aeromonas* spp. One was CBB for *A. salmonicida*, cause of fish furunculosis; these plates were incubated at 22 °C; suspect blue colonies were transferred to TSA. The other medium (SGAP-10C) was for growth and enumeration of motile *Aeromonas* spp. (Huguet and Ribas 1991, Jenkins and Taylor 1995). Although other organisms may grow on SGAP-10C, such as *Pseudomonas fluorescens*, they are easily distinguished from *Aeromonas* spp. by colony characteristics and additional biochemical tests, such as fermentation of glucose. *Pseudomonas* isolation agar (PIA; Difco Laboratories, Detroit, MI) was for growth of *Pseudomonas* spp., of which many members of this or related genera are commonly found in aquatic environments or as part of the flora of healthy fish. *Edwardsiella* isolation medium (EIM; Shotts and Waltman 1990) is both differential and selective and was used for *Edwardsiella ictaluri*, cause of enteric septicemia of

catfish, and *E. tarda*, a potential pathogen often found in warm- and cold-blooded animals. Selective *Cytophaga* agar (SCA; Hawke and Thune 1992) was employed as a selective medium for *Flavobacterium columnare*, these plates were incubated at 37 °C for additional selection.

Challenge Procedures: Mussels and Fish

For the bacterial challenges of mussels, 20 tanks (27-L each) were used, each containing 10 mussels. The 200 animals, listed previously, were distributed equally by species among the 20 groups. The animals were allowed to acclimate in the tanks for 2 d before challenge. Sixteen of the tanks received bacteria: this included 14 that were of the predominate normal flora from rainbow trout from the White Sulphur Springs Hatchery, seven that were originally isolated on BHIA, and seven from CYTO. Another two tanks were challenged with the known fish pathogens *A. salmonicida* and *R. salmoninarum*, and there were four control groups exposed, one each for the four sterile media used to grow the challenge bacteria. The challenge strain of *A. salmonicida* originated from a furunculosis-diseased Atlantic salmon (*Salmo salar*) from Vermont, and the *R. salmoninarum* isolate (ATCC33209) was from a Chinook salmon (*Oncorhynchus tshawytscha*) from Oregon. The normal flora bacteria were each grown in a 200-mL quantity of either BHI broth or CYTO broth, the same medium that was used for original isolation. *Aeromonas salmonicida* was grown in 200 mL of TS broth, and *R. salmoninarum* was grown in 200 mL of KDM2 broth. Cultures were grown for 48 hours, except for *R. salmoninarum*, which was grown for 14 days. A viable cell count was done for each culture, and this was determined by preparing sets of serial tenfold dilutions in the homologous broth medium, and drop inoculating known quantities on plate media. Colonies were quantified, and the number of cfu/mL of tank water at the start of the challenges was calculated. To perform a challenge, the water supply to the tank was turned off, the culture was poured and mixed in, and exposure was for 24 hours. Then, the water was turned on and allowed to rinse for 24 hours; then to each tank, 10 fish were added: five rainbow and five brook trout. The mussels and fish were then observed for a period of 21 days for mortality and development of abnormal signs or pathology. Kidneys of fish that succumbed were cultured onto the appropriate medium, and subsequent bacterial growth was identified to confirm re-isolation of the bacterium used to challenge the mussels.

RESULTS

Physical data on the animals collected for isolation of bacteria are presented in Tables 1 and 2. The predominate bivalve species collected for the six trials was *A. plicata*, accounting for 81.5 % of the total. The average weight of the animals varied, depending on species, and ranged from 25.7 g for the single *E. lineolata* to 294.7 g for the *A. plicata*. However, regardless of over-all size, the percentage of the total weight comprised of soft tissues ranged from 10.5 to 15.2 %. Of the total soft tissues, the ratios of our portioned gut to OT samples were also similar among the hosts. The percentage of soft tissues collected as gut samples ranged between 53 to 64 %, with the paired OT samples comprising the balance of the total soft tissues. With exception of trial 4, the distribution of *A. plicata* physical data remained relatively similar throughout the season (Table 2). In five of the trials, the mean weights of the *A. plicata* ranged from 247.6 to 294.7g; whereas, with trial 4, the average weight was less, 153.7 g.

Results of the bacteriological analyses including enumeration of bacterial flora, numbers and qualitative characterization of suspect colonies, DFAT staining for *R. salmoninarum* in tissue homogenates and Ohio River surface water temperatures at the time and location of collection for the six trials are given in Table 3. Total bacteria counts using the routine growth media BHIA and

TABLE 3.

Mean bacterial counts (cfu/g), colonies selected and characterization results of suspect fish pathogenic bacteria isolated from freshwater bivalves sampled six times (Trials 1–6) during the 1997 collection season. The data are mean or summary values for the number of animals per trial. Trials 1–5 had 20 animals each, trial 6 had eight.

Medium[a]	Trial 1 (29 °C[b])	Trial 2 (27 °C)	Trial 3 (24 °C)	Trial 4 (21 °C)	Trial 5 (20 °C)	Trial 6 (10 °C)
BHIA	1.07×10^5	2.90×10^5	2.31×10^5	2.10×10^5	2.85×10^5	2.01×10^4
CYTO at RmT	1.81×10^5	4.99×10^5	2.91×10^5	4.29×10^5	2.73×10^5	1.07×10^5
SGAP-10C	3.81×10^4	8.39×10^4	3.77×10^4	2.03×10^4	8.07×10^4	2.95×10^3
PIA	8.37×10^3	5.41×10^4	7.61×10^3	6.69×10^3	2.01×10^4	3.36×10^3
CYTO at 15 °C	NS[c]	NS	1 (0)[d]	14 (0)	NS	NS
SW/ROD	18 (0)	12 (0)	21 (0)	21 (0)	21 (0)	10 (0)
CBB	3 (0)	12 (0)	11 (0)	13 (0)	12 (0)	7 (0)
EIM	6 (0)	10 (0)	7 (0)	8 (0)	7 (0)	8 (0)
SCA	NS	NS	NS	NS	NS	NS
ROGO	NG	2, 1.2×10^2[e]	NG	NG	2, 8.0×10^1	1, 8.0×10^1
ABA	3, 5.90×10^4	1, 3.20×10^2	NG	NG	NG	NG
SKDM	9 (0)	NS	9 (0)	14 (0)	12 (0)	12 (0)
DFAT	3: 3, 1, 40[f]	2: 1, 1	2: 1, 5	3: 2, 1, 1	8: 2, 1, 1, 1, 1, 1, 1, 1	1: 1

[a] Media for total counts: BHIA, brain heart infusion agar; CYTO, *Cytophaga* agar; SGAP-10C, for motile *Aeromonas* spp.; PIA, *Pseudomonas* isolation agar. Gram-negative media: CYTO@15 °C, for *Flavobacterium psychrophilum;* SW/ROD, two media for *Yersinia ruckeri;* CBB, for *A. salmonicida;* EIM, *Edwardsiella* isolation medium; SCA, for *F. columnare*. Gram-positive media: ROGO, primarily for *Lactobacillus* spp.; ABA, azide blood agar; SKDM, for *Renibacterium salmoninarum;* DFAT, direct fluorescent antibody test for *R. salmoninarum* of 1:2 dilution of each tissue.

[b] Temperature of Ohio River surface water at time and location of collection.

[c] NS = bacterial growth, but no characteristic colonies present. NG = no growth.

[d] Number of suspect colonies picked for biochemical characterization or for SKDM, DFAT for *R. salmoninarum* (number positive).

[e] Number of samples with growth, mean cfu/g of those with growth.

[f] Number of tissues positive: number of positive cells per 100 microscopic fields of each positive sample.

CYTO remained quite similar throughout the sampling season, with exception of trial 6 for which counts were less, presumably because of the colder water temperature. The average bacterial counts on BHIA ranged from 2.01×10^4 to 2.90×10^5 cfu/g of soft tissue and for CYTO, the range was 1.07×10^5 to 4.99×10^5 cfu/g. The range for *Aeromonas* spp. isolated on SGAP-10C was 2.95×10^3 to 8.39×10^4 cfu/g; whereas, for *Pseudomonas* spp., counts on PIA were between 3.36×10^3 and 5.41×10^4 cfu/g of soft tissue. All of the *Aeromonas* spp. were presumptively identified as being of the motile *Aeromonas* spp. group, because the only member of this group that is nonmotile and considered significant with regard to fish disease is *A. salmonicida* and none of the suspect blue colonies transferred and characterized off CBB were nonmotile. The sum of bacterial counts off SGAP-10C and PIA accounted for a large portion of the total bacteria. The water temperature was lowest when animals were collected for trial 6, not only had the animals burrowed beneath the surface of the river bottom and limited the number collected but also resulted in the lowest counts for total bacteria, motile *Aeromonas* and *Pseudomonas*. Conversely, the highest cfu/g of tissue for these three groups of bacteria were all recorded on trial 2, when the water temperature was near the highest at 27 °C. The proportion of the total bacteria that was comprised of motile *Aeromonas* and *Pseudomonas* was also highest from trial 2.

A number of Gram-negative colonies suspected of being pathogenic for fish were selected off the media CYTO at 15 °C, SW/ROD, CBB, and EIM (Table 3). Suspect colonies were identified based on meeting criteria set forth in the specific references. After transfer and biochemical characterization, none was confirmed as being a fish pathogen. There were no characteristic *F. columnare* colonies cultured from any tissues of any of the trials. In a previous year this bacterium was isolated from an *A. plicata* that came from the same location in the Ohio River (Starliper et al. 1998). The selective Gram-positive isolation media, ROGO and ABA, supported very minimal growth relative to that of the media used for Gram-negatives. Bacterial growth was present on ROGO medium in three of six trials, but from only five of the 96 tissue samples assayed. The counts of the tissues with growth ranged between 8.0×10^1 and 1.2×10^2 cfu/g. Tissues from two of the trials showed growth using ABA medium and these were from only four of 80 samples with averages of 5.90×10^4 and 3.20×10^2 cfu/g for trials 1 and 2, respectively. Isolates selected from the ABA culture plates were also identified as α–hemolytic *Lactobacillus* and were catalase negative, and, therefore, differed from *Carnobacterium piscicola* (formerly *Lactobacillus piscicola*) that has been previously noted to cause disease and low mortality primarily in postspawning rainbow trout (Starliper et al. 1992). Only in trial 2 was there any bacterial growth of any tissue samples on both ROGO and ABA. Serological based observation of the 1:2 tissue homogenates for *R. salmoninarum* using the DFAT showed positive fluorescent cells of correct morphology from every trial. Trial 5 had the largest number of tissues (8 of 40) with at least one cell detected within 100 microscopic fields of view. The number of cells detected in those positive tissue samples was either one or two for most, but 40 cells were seen in 100 fields from one *Q. quadrula* OT sample. However, no bacterial colonies yielding fluorescent cells having correct morphology for *R. salmoninarum* were noted on SKDM primary isolation medium from these or any other tissue homogenates. One suspect bacterial colony from SKDM growth plates did yield excellent positive fluorescence, but the individual cells were too large to be considered of correct *R. salmoninarum* cell morphology; this was from a *Q. metanevra* OT homogenate.

At the start of the 24-hour bacterial challenges, the average number of viable bacteria was 5.31×10^6 cfu/mL of tank water for the seven groups of freshwater bivalves exposed to the selected normal flora bacteria isolated from fish and grown in CYTO media. There was an average of 4.89×10^6 cfu/mL of challenge tank water in the seven groups of animals exposed to the normal flora bacteria isolated and grown in BHI medium. There was 1.98×10^6 cfu/mL of tank water in the group exposed to *A. salmonicida* and for *R. salmoninarum*, there was 3.53×10^5 cfu/mL. During the actual 24-hour waterborne exposures, there was no mortality experienced in the bivalves. The only bivalve death in the duration of the study, a *Q. quadrula*, occurred on day 8 of the 21-day cohabitation with fish and was in the group exposed to the TSB medium control. There was no mortality in the brook or rainbow trout in any groups that were placed to cohabit with mussels previously challenged with the 14 bacterial types cultured from the White Sulphur Springs National Fish Hatchery. Also, there were no deaths of fish in the *R. salmoninarum* group or the four control groups exposed to bacteriological media only. However, in the group of animals challenged with *A. salmonicida*, mortality in brook trout began on day 8 of cohabitation, two more died on day 15, and the remaining one on day 21. One rainbow trout died on day 21. Of these dead fish, there were no external lesions produced, but there was extensive internal pathology indicative of a systemic, Gram-negative bacterial infection. There were elevated amounts of red, ascitic fluid, hemorrhaging of internal organs, and the hind gut was filled with yellow, pus-like material. Kidney tissues of dead fish inoculated onto CBB plates resulted in heavy growth with presumptive blue *A. salmonicida*. Single colonies were picked, and their identity was characterized as *A. salmonicida* with the following criteria, in addition to blue on CBB: production of brown, water soluble pigment on TSA, K/A on triple sugar iron agar, oxidase positive, nonmotile by the hanging drop method, gelatin liquefaction positive, and a negative ornithine decarboxylase. The greater mortality in brook trout was expected, because they are known to be more susceptible to *A. salmonicida* than are rainbow trout (Cipriano 1982). After the 21-day observation, kidney tissues of the surviving rainbow trout were streak plated onto CBB, and *A. salmonicida* was isolated and the identity confirmed, as previously, from two of the fish. The 10 mussels in this group were comprised of seven *A. plicata* and one each of *Q. metanevra*, *P. cordatum* and *Q. quadrula*. After the 21 days of cohabitation, the gut and OT soft tissue samples of each of these animals was excised, homogenized, and diluted as previously described with drop inoculation onto CBB plates for isolation of *A. salmonicida*. From these, no suspect colonies were cultured from any of the 20 soft tissue homogenates; therefore, *A. salmonicida* was not reisolated.

The 10 freshwater bivalves challenged with *R. salmoninarum* included seven *A. plicata*, two *O. reflexa*, and one *Q. pustulosa*. In contrast with the group challenged with *A. salmonicida*, no mortality in fish occurred. After the observation period, the fish kidney tissues of surviving fish were used to prepare smears on microscopic slides for evaluation by DFAT. No fluorescent *R. salmoninarum* cells were detected. At this time, the same was done for the gut and OT tissues of the ten mussels, and one *A. plicata* was positive with two cells of correct morphology for *R. salmoninarum* being detected in 100 microscopic fields viewed. However, it is not known if these cells were alive, beecause the DFAT stains both

live and dead cells. Upon observation of the slides prepared from the mussel tissues, there was a significant amount of small (1μ in diameter) fluorescent particles present that did not have uniform shape and were not confused with intact cells. There were too many, and each was too small to be quantified accurately. It is not known if these entities in some way originated from the *R. salmoninarum* cells used for challenge or if they were artifactual staining. In either case, this is unique, because this is not typically noted on stained slides prepared from fish kidneys, whether the tissue is positive or not for the bacterium.

DISCUSSION

With intensive fish culture and fish health management, the best defense against pathogenic diseases is prevention (Piper et al. 1982). All of the major bacterial pathogens of salmonid fishes may involve horizontal transmission. This might occur among resident individuals within a facility or could result from introduction of a pathogen to resident fish by contamination with a new lot of fish placed into the facility. Relocated fish could be carriers of a pathogen and because of their past exposure may have some innate immunity that would allow them to harbor an organism and not display any obvious signs of disease. Then, when naive fish are exposed to bacteria shed by the carriers, an epizootic may ensue. Because of this potential, it is imperative that fish to be relocated undergo a health inspection to identify pathogens, including those that are not obvious because of a lack of clinical signs. The potential for introduction of pathogens via relocated freshwater bivalves is also of concern. Animals may be originating from open and uncontrolled environments, such as the case with those from the Ohio River, where they might be exposed to wild fish that could be diseased. Bacteria are shed into the environment and the bivalves could uptake bacteria either as a food source or by simply filtering contaminated water. The primary pathogens of salmonid fishes are not known to cause diseases in freshwater bivalves; therefore, animals would be unlikely to become carriers of a pathogen in the sense that susceptible fish can following their survival of an epizootic. Once freshwater bivalves are removed from the source (shedding) of a bacterial pathogen and relocated to a pathogen-free water supply, such as could be the case of quarantine for zebra mussels, the length of time the pathogen remains present and viable in the tissues and could still be infective to fish is not known. This is especially important considering the results of the cohabitation study where mussels were exposed to *A. salmonicida*. In a study by Plusquellec et al. (1994) with two marine bivalves, the mussel *Mytilus edulis* and the oyster *Crassostrea gigas*, retention of the enteric human pathogenic *Salmonella* following artificial exposure and air drying showed retention of viable cells of at least 5 days at 12 to 15 C and as long as 20 days at 10 C. However, when the clam *Mercenaria mercenaria* was artificially exposed to *Escherichia coli* or *S. typhimurium*, and the infected animals were moved to flowing, pathogen-free seawater, they are able to reduce viable cell loads in tissue homogenates within 24 hours by factors of 10^4 and 10^5, respectively, from a starting tissue load of between $1–2 \times 10^5$ cfu/g (Timoney and Abston 1984). A change in bacterial flora was similarly noted within 24 hours in freshwater bivalves that were not initially exposed artificially to bacteria, rather these animals were simply relocated from one water supply to another (Starliper et al. 1998). In this study, the total bacterial count per gram of soft tissue remained quite stable at both water sources, but the most notable change was a relative increase of nonfermenting bacterial types, coinciding with a decrease in other bacterial types after 24 hours of being in the different water supply.

The bivalves that were collected in trial 4 were smaller than those collected in the other five trials (Table 2). The smaller animals were several years younger than the larger ones. When collecting animals from a large environment, such as the case with the Ohio River, a group with size characteristics unique to other groups collected is not unusual. Bivalves are not evenly deposited on the river bottom, rather, they often occur clustered as to age and species, which has to do with the movement of the host and where the juveniles drop from the host. This is particularly relevant with *A. plicata*, because of the variety of fishes that may act as hosts and include many species of the family *Centrarchidae*, sauger (*Stizostedion vitreum*), and the flathead catfish (*Pylodictis olivaris*).

In the study by Starliper et al. (1998), *Flavobacterium columnare*, cause of columnaris disease in many cool and warm water fishes, was isolated from an *A. plicata*. This animal was assayed directly after being removed from the Ohio River and had not been placed into pathogen-free water for any time prior to analysis. The pathogen was not isolated from animals that were collected at the same time, and location but had been in pathogen-free, flow-through water for 24 hours. Alone, information on the isolation of a fish pathogen from a bivalve that could be intended for relocation to a salmonid rearing facility is discouraging. However, before relocation, if they undergo quarantine according to a protocol such as that of Gatenby et al. (1998) to ensure that zebra mussels are not inadvertently spread and if the animals are maintained in pathogen-free water, there is the chance that pathogens may be depurated during the quarantine. This is surmised from the demonstrated rapid change in flora after being moved to a different water supply and because *F. columnare* was not isolated from animals after having been relocated for 24 hours.

The detection of cells of correct morphology for *R. salmoninarum* from the DFAT of mussel tissue homogenates could become a concern for fish health managers of salmonid rearing facilities. This bacterium and the disease it causes, when in a population of fish, presents significant health management problems, because a primary means of pathogen transmission is vertical, in addition to the fact that it is horizontally transmitted. Therefore, presence of any fluorescent bacterial cells of correct morphology noted in fish kidney tissues or ovarian fluids of spawned fish is not good, particularly at those facilities that are involved with egg production and shipment. The nature of the bacterium's being very slow growing and difficult to isolate, especially in low numbers, only adds to the dilemma of fish health managers faced with the situation. When fish health inspections yield only minimal numbers of positive cells using the DFAT or minimal positive results from other serology-based assays in the absence of bacterial isolation or clinical disease signs in fish, management personnel are faced with difficult decisions on the health status of the population. Therefore, if relocated mussels might be determined to be a source of a bacterium that could result in positive cells by DFAT, regardless of a lack of culture isolation, a significant concern is realized. An important topic for further study would be to evaluate if a 30-day quarantine in a clean or different water supply would result in depuration of cells that may yield fluorescence.

In the six trials of the present study, the results using the DFAT and culture on SKDM for detection of *R. salmoninarum* from bivalve tissues were not in agreement; no cells were noted from culture; whereas, there were using the DFAT for tissues. Discrepancies of this sort are not uncommon using various methodologies for detection of this bacterium in fish (Cipriano et al. 1985; Teska et al. 1995). A number of factors contribute to this and include the slow and difficult nature of isolation and growth of *R. salmoni-*

narum, specificity and sensitivity of antisera, the host, and the extent of infection within a population of fish. Furthermore, the DFAT was originally developed as a method for quick, presumptive diagnosis of clinical bacterial kidney disease (Bullock et al. 1980) that would later be confirmed by bacterial culture. The advantage was that a DFAT could be done in hours, as compared to weeks for primary isolation. This affords quick intervention for fish health managers to control the disease and prevent further spread. With heavily infected fish, the two methodologies correlate well, but when used for relatively healthy, pathogen carriers, the agreement may decrease.

In the cohabitation group with mussels previously exposed to *A. salmonicida* and brook and rainbow trout, it is noteworthy that after 21 days, the bacterium was not re-isolated from the mussels' soft tissues. This is interesting, because there were infected and dying fish present in that tank water, and clinically diseased and carrier fish are known to shed viable *A. salmonicida* cells. However, with only four rainbow left at the end, the quantity of bacteria shed might have been too few to maintain bacterial presence within the *A. plicata*. Also, the normal flora of the mussels may have displaced and/or prevented further infection, because to mussels, it is assumed that *A. salmonicida* is merely an environmental organism and not pathogenic.

With the *A. salmonicida* challenge experiment, it is encouraging that after being exposed to a load of viable bacteria in the water that is greater than would occur naturally, the bacterium was not isolated from animals after the challenge's observation period. Again, it would be important to evaluate if the quarantine for 30 days to eliminate zebra mussels is sufficient for native bivalves to concurrently depurate fish pathogens. Additional topics for research are to extend beyond 24 hours the duration between when mussels are removed from bacterial exposure and when susceptible fish are introduced. Furthermore, the bivalves can be exposed to lesser bacterial cell concentrations and a more natural challenge method, those more analogous to what might be encountered in nature and then evaluate contagion. Also, a determination should be made on whether the bacterium enters the soft tissues or merely is contained in the fluid portion outside the soft tissues, but within the shell.

Following the challenge and cohabitation involving *R. salmoninarum* and when fish and mussels tissues were evaluated by DFAT, no cells were detected in fish, and only two were detected from one *A. plicata*. These results were not surprising for this bacterium. Reproduction of experimental bacterial kidney disease in a laboratory by contact exposure is very difficult and requires significant effort and time. Wolf and Dunbar (1959) were able to produce mortality in brook trout by a noninjectable challenge with *R. salmoninarum* only after fish were maintained in tanks with bricks in place as a means for abrasion, then the water level was dropped daily and viable cells were added. Still, it took 96 days for the first death. In another study, IP injection of brook trout with a number of viable cells similar to that per mL of tank water used in the present study, the mean days to death for the group of fish was about 28 days, and the first occurred on about day 25 (Starliper et al. 1997). Because horizontal or contamination infection to fish is difficult and because this bacterium has a limited host susceptibility range that is known to include primarily salmonid fishes, perhaps the chance for mussels to act as vectors is remote. Particularly, if mussels are able to rid the bacterium as noted in the present study when exposed to *R. salmoninarum*, that after 21 days in clean water only two cells were detected by DFAT of the soft tissue homogenates. Furthermore, the high frequency of fluorescent particles in the tissues might be indicative of cellular debris resulting from the bacterial cells being digested by the mussels.

All of the freshwater bivalves in these studies were used, because they are readily available, and they exist in high numbers; however, they may not be species that will be collected as part of the relocation program. Once techniques are developed using common animals and are available, the appropriate studies may then be repeated using minimal numbers of surrogates selected to represent those animals that do fit the criteria for relocation.

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DEVELOPMENT OF ARTIFICIAL LOBSTER BAITS USING FISH SILAGE FROM TUNA BY-PRODUCTS

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ABSTRACT The present work shows that an artificial bait can replace the traditional baits (raw fish) using tuna by-products preserved with citric and phosphoric acids to produce an acid fish silage. After 2 months preservation by mixing minced viscera from the tuna industry with 2.6% each of citric and phosphoric acids, the silage was ready to be blended in two different mixtures; acid, or non-neutralized, and neutralized with 5% sodium carbonate to reach a pH close to 6. The baits were made by mixing both types of silage (non-neutralized and neutralized) with starch, fishmeal, and fish oil to prepare a sausage-like bait weighing 150 g each. A cellulose membrane was used to cover the bait and a cotton string to close them at both ends. After measuring the soluble protein loss at different times throughout 48 h, it was shown that baits leach out up to 0.88 mg protein per g bait per hour. Both types of bait did not show any significant difference compared to the natural bait (raw fish) when tested under commercial fishery conditions. The baits were also tested for dehydration by hanging until a 14% water content was reached. However, the dry baits showed a lower leaching rate when they were exposed to water. More experiments should be performed in order to conclude if dried baits are able to attract lobsters in a way similar to the moist baits.

KEY WORDS: *Panulirus*, lobster, artificial baits, baits

INTRODUCTION

The lobster is an economically important resource of Mexico, especially in Baja California, their principal area of capture (Briones and Lozano 1994). According to the Mexican foreign trade bank (Bancomext), 2,552 tons of lobster was captured in 1997 in Mexico, where the spiny lobster (*Panulirus interruptus*) was the most important species, which valued 22 million dollars. This placed lobster sixth in economic importance of Mexican export fishery products of 1997.

The fishery of lobster in Baja California it is one of the oldest fisheries in the region. In 1959, the fishery was given in concession to 19 cooperatives along the coast. In 1992, the private sector was allowed to catch lobster with a governmental permission or concession (Diario Oficial de la Federación 1992), but the lobster production still remained mainly cooperatives-controlled. Lobsters are caught from September to February, depending on the reproductive status of the animals. The lobsters are taken in traps, which usually contain two entrances and one compartment for the bait. Lobsters are nocturnal; therefore, traps are checked daily in the early morning during open season, at which time, the lobsters are removed, and the bait is replaced.

Bait is very important for this activity, because the fishermen depend upon a regular and quality supply. Bait generally consist of fresh or frozen fish, which is obtained either by the head office in the cooperative or the by fishermen, where sometimes a whole day is devoted to obtaining sufficient bait for the following day. They act as attractants by the leaching from the hydrolyzed protein or fluids from fish (Ache et al. 1978), which is sometimes increased by bacterial decomposition (Zimmer-Faust 1987; Zimmer-Faust and Case 1983; Zimmer-Faust et al. 1996). The fishermen choose their type of bait according to their prior experience with different fish or invertebrate species or size of the bait, which often results in longer periods of time to get their favorite bait. Cans with fish in tomato sauce are also sometimes used, where small holes at both sides of the can are made leaving the can for one or two days.

Baits are one of the main problems faced by this fishery since large quantities of fish must be stored in short term refrigeration facilities, resulting in large expenses for the co-operatives. One of the main co-operatives of the region estimate that up to $50,000 U.S. are used for baits during the season (Jacobo Castro, Cooperative head officer, pers.com.). Moreover, good quality fish suitable for human consumption are often used as bait, which has other implications. According to FAO demand for fish products is increasing and the use for certain products should be destined only to humans (FAO 1997). Besides the problems above mentioned the handling of fresh or frozen fish has considerable energy demands and also requires that fishermen collect their bait daily from the co-operative office. Fishery camps are sometimes far from the storage rooms from the head office, which makes this type of handling difficult.

Several artificial baits have been tried in order to substitute the fresh and frozen fish (Mackie et al. 1980; Carr 1986; Daniel and Bayer 1987; Miller 1990 and Mohan-Rajan and Shahul 1995), but for some reasons non-of them are commercially available. In the present work a new concept of bait is introduced, which uses tuna by-products preserved as acid silage extruded in a cellulose membrane.

MATERIAL AND METHODS

Baits Preparation

Tuna by-products were ensiled as described by Viana et al. (1993). In summary, chopped tuna by-products were mixed with 2.6% phosphoric acid, 2.6% citric acid and 0.1% sodium benzoate was added as a preservative. The mixture was blended to obtain a homogenate and left for 60 days in plastic buckets, while adjusting the pH lower to than 3.5. Further, the silage was separated in two

groups and one of them was neutralized using 5% sodium carbonate as described by Raa and Gildberg (1982) prior to cold mixing with the other ingredients as described in Table 1. The sausages were extruded in a pasta Machine (Rosito Bisani TR110) using a funnel and cellulose membrane. The sausages were tied with cotton string at both ends every 15 cm long, and were hang or frozen until used.

Lab analysis

Protein leaching

The baits were tested for protein leaching at 16 °C by immersing 30g baits in triplicate in 250 mL beakers containing 100 mL seawater at constant turbulence created by a horizontal lab mixer. A 1 mL sample of seawater was taken at 0, 6, 12, 24, 30, and 48 h and soluble protein content was determined. Soluble protein was estimated following the Lowry method (Lowry et al. 1951), using bovine serum albumin (BSA) for the calibration curve. The amount of soluble protein reported is expressed as mg protein equivalent to bovine serum albumin (BSA) per g bait.

Dry matter loss

The remaining dry matter was measured at constant dry weight by recovering the baits after 0, 6, 12, 24, 30, and 48 h immersion in seawater. Percentage dry weight was calculated as the dried residue weight of triplicate samples of each bait after drying to constant weight at 100 °C during 24 h.

Total protein

Total nitrogen was determined using triplicates samples analyzed by the Kjeldahl method (AOAC 1995). Crude protein was calculated as % N × 6.25.

Microbiological analysis

Baits were tested for bacterial content under storage at days 0, 8, 16 and 183. Baits samples were stored in plastic bags at 3–4 °C and total bacterial content was determined (Aerobic plate count APC) following the procedure described by the FDA-AOAC (1992).

Dehydration / Re-hydration tests

In order to determine the behavior of baits under storage, the baits were dehydrated when hanged at 3–4 °C until 15% humidity was reached. The dry baits were tested for time of re-hydration and tested for protein leaching as described before.

TABLE 1.

Ingredient composition of the artificial baits tested in this study, given as percentage of inclusion either wet (silage) or dry matter.

Ingredients	Tuna By-Product Silage
Tuna by-products silage[a]	70.9
Fishmeal[b]	15.0
Modified corn starch	10.0
Fish oil	4.0
Sodium benzoate	0.1

[a] Made from the cannery industry, kindly provided by Rowen SA, Mexico.
[b] Kindly provided by Proesa Ensenada, Mexico.

TABLE 2.

Proximate composition of the artificial baits tested in this study, given as percentage.

Ingredients	Tuna By-Product Silage (%)
Protein (g/100g)	27.4
Total lipids (g/100g)	11.9
Total solids (g/100g)	46.1

Fishing experiment

Both neutralized and non-neutralized fish silage baits were tested in the Co-operative of Emancipacion, BCS, Mexico during 6 days in January, 1996. For this, 150 neutralized and 60 non-neutralized baits were prepared while only 41 neutralized and 12 acid baits were tested in pairs together with a traditional bait (fish parts). Each bait (artificial or traditional) was introduced separately to traps in the early morning (5:00 AM) and 24 h later the number and size of lobster were registered.

Statistical procedures

A one way ANOVA (Zar 1999) was used to compare between baits, non-neutralized vs. neutralized tuna-fish silage. To test possible differences between treatments on leaching and stability at different levels of time an analysis of covariance (ANCOVA) was used according to Sokal and Rohlf (1981). To test the baits functionality in the lobster traps, a one way analysis of variance (ANOVA) was used to compare between non-neutralized and neutralized fish silages and traditional bait. The computer package of Sigma-Stat for windows, version 1.1 (Jandel 1994) was used in these statistical analyses.

RESULTS

The lobster baits showed a proximate composition of 27.4% crude protein, 11.9% total lipids and 46.1% total solids (Table 2). When the neutralized and non-neutralized baits were tested for leaching of protein no significant differences were observed be-

TABLE 3.

Leaching of soluble protein.

Leaching Time (h)	Neutralized Tuna By-Product Silage (mg BSA/g)	Non-neutralized Tuna By-Product Silage (mg BSA/g)	*P* ANOVA
6	7.7 ± 0.77	12.0 ± 1.66	0.114
12	11.4 ± 0.46	16.5 ± 3.97	0.200
24	28.8 ± 2.02	23.6 ± 1.03	0.061
36	36.6 ± 2.03	31.8 ± 3.33	0.259
48	39.4 ± 2.54	36.5 ± 4.41	0.583
Leaching rate (mg/g/h)	0.84	0.88	

Covariance Table **n = 40** **R = 0.895** **R^2 = 0.802**

Source of variation	SS	df	MS	Fs	P
within groups	5.005	1	5.005	0.16	0.691
Time (X)	4667.579	1	4667.579	149.587	0.000
Error	1154.516	37	31.203		

Mean values obtained in neutralized and non-neutralized tuna by-products baits. Covariance analysis is indicated below.

TABLE 4.

Dry matter loss.

Time (h)	Dry Matter Loss of Nonneutralized (%)	Remnant Total Prutein After Baits Were Immersed in Seawater at Different Times (%)
0	0	27.46 ± 0.23
6	ND[1]	27.08 ± 0.12
12	2.05 ± 0.682	26.03 ± 0.04
24	5.67 ± 1.07	24.24 ± 0.24
36	8.25 ± 0.307	23.85 ± 0.37
48	14.88 ± 0.827	22.24 ± 2.70
Rate loss (g/100g/hr)	0.14	0.11

[1] ND, not determined.
Mean values of baits immersed in water at different times.

tween treatments at any time up to 48 h, as shown in Table 3. By a covariance analysis it was also corroborated that no differences were found in the rate of leaching being 0.84 and 0.88 mg/g/h, respectively.

The dry matter loss on non-neutralized baits showed a rate loss of 0.14 grams per 100 grams per hour up to 48 h. The loss for the first 24 h was below 5% (Table 4). Moreover, by measuring the remnant total protein it was shown a decrease in protein content over time, from 27.5% to 22.2% after 48 h with a rate loss of 0.11 g protein per 100 g per hour (Table 4).

The dehydration of baits to a constant 14.3% water content was reached after 40 days from the original of 54.1% (Table 5). Moreover, those dehydrated baits showed a significantly lower leaching of protein after being re-hydrated compared to the fresh bait (Table 6) with a leaching rate of 0.55 mg protein per gram bait per hour, compared to 0.88 observed in the fresh bait. Non-neutralized baits showed no significant growth of undesirable bacteria with a growth of less that 25 colonies per gram even after 183 days.

The Table 8 shows the results obtained in the field with traditional baits (fresh mackerel) compared to the non-neutralized and neutralized fish silage baits. No differences were detected between all three different groups with the lobster at commercial size. However in the small lobsters group (within the illegal size) differences were observed where the non-neutralized bait showed less attractant activity followed by the other two treatments, the non-neutralized and the traditional baits.

DISCUSSION

Tuna by-products are rarely used in Mexico, resulting in large amounts of products, which are often discharged, causing environmental problems in nearby cities. However, although fish silage has proven to be a good product as an alternative to fish by-products (Raa and Gildberg 1982), its production in Mexico has not been established. Notwithstanding, fish silage has been described as an ingredient in abalone feed formulation (Viana et al. 1996). The present work demonstrates that fish silage can be effectively used as an ingredient to formulate lobster baits without observing any significant difference compared to the fresh fish regularly use in Mexico as bait.

Fishermen use a piece of fish every day, which can weight between 500 g to 1 kg (Raul Celis, pers. comm.). This piece is changed every day in order to be attractant to lobster. The baits can be supplied from the Cooperative's head office or by the own fisherman. As stated before it is difficult to estimate the cost for bait use in the season for all co-operatives, but they need to change opinion to a better opportunity in order to be able to stand the market price fluctuations.

The fish silage baits presented here are easy to use and the price would be far less if fish by-products are used. No differences were observed in leaching and as attractants between neutralized and non-neutralized baits (0.84 and 0.88 mg protein/g/h, respectively), suggesting that neutralization with 5% sodium carbonate is not necessary. This means that production of bait will require fewer procedures while conserving the bait at low pH. Bacteria grow very easily in fish products due to their highly digestible protein and soluble carbohydrates. At lower pHs as in the acid silage it is known that essentially no bacteria will grow (Raa and Gildberg

TABLE 5.

Mean values for water content in baits left at 4 °C until 40 days.

Time (Days)	Tuna Baits (Humidity %)
0	54.11
4	34.29
10	27.31
17	18.61
27	16.72
35	15.07
40	14.31

TABLE 6.

Mean values of protein leaching obtained in dehydrated baits either neutralized and non-neutralized. Cuvariance analysis is indicated below.

Time (h)	Fresh Bait (mg BSA/g)	Dehydrated Bait (mg BSA/g)	P
6	12.0 ± 1.66	4.71 ± 1.06	0.00
12	16.5 ± 3.97	11.49 ± 0.36	0.20
24	23.6 ± 1.03	14.53 ± 1.50	0.00
36	31.8 ± 3.33	19.59 ± 2.19	0.02
48	36.5 ± 4.41	21.53 ± 0.47	0.01
Leaching rate (mg/g/h)	0.88	0.55	

Covariance Table **n = 40** **R = 0.884** **R^2 = 0.781**

Source of variation	SS	df	MS	Fs	P
within groups	941.719	1	941.719	39.495	0.000
Time (X)	2203.992	1	2203.992	92.434	0.000
Error	882.223	37	23.884		

TABLE 7.

Total bacteria count in lobster baits at different times.

Time (Days)	Neutralized Tuna By-Product Silate (colonies/g)
0	<25
8	<25
16	<25
183	<25

TABLE 8.

Average value of lobsters caught per trap under experimental conditions.

Variable	Fresh Bait (Mackerel) (n = 41)	Neutralized Tuna By-Product Silage (n = 41)	Non-Neutralized Tuna By-Product Silage (n = 15)
Small lobster (illegal size)	16.32 ± 1.58[a]	12.63 ± 1.79[a,b]	6.91 ± 3.32[b]
Commercial lobster (legal size)	1.15 ± 0.16[a]	1.02 ± 0.19[a]	1.00 ± 0.35[a]
Total	17.47 ± 1.65[a]	13.66 ± 1.87[a,b]	7.92 ± 3.46[b]

Significant differences are indicated with different letters for each variable ($P < 0.015$).

1982). However, when the acid fish silage is combined with other feed ingredients the pH in the mixture approaches 6 depending of the type of ingredients (Rivero and Viana 1996). In the present work the non-neutralized baits mixed together with fishmeal and starch at low proportions bacteria failed to grow even after 183 days (< 25 colonies/g), which means that the presence of non-neutralized silage could effectively inhibit decomposing bacteria. This is considered of particular importance since food for human consumption needs to be free of microbes, it will be necessary to maintain baits and lobster under hygienic conditions during their fishery.

When baits are hang they can be reduce to humidity as low as 14% after 40 days. Even if leaching was observed after re-hydration, the amount that washed out was significant lower (0.55 compared to 0.88 mg protein/g/h). In the present work it was not tested the effect of dry baits as attractants and therefore it will be necessary to performed another experiment in order to conclude their effectiveness. In the meantime, baits can be packed in vacuum bags to avoid dehydration if longer periods of time are necessary to store without refrigeration as was done in the bacteria growth test. Both neutralized and non-neutralized baits performed similarly in terms of leaching, dry matter loss and for catching legal size lobster. However, in the field trial, small lobsters (illegal size) showed to be less attracted by the non-neutralized than that of the neutralized fish silage and traditional baits (6.91 compared to 12.63 and 16.32 lobster/trap, respectively). The reason is difficult to explain, but it could also be an advantage to catch fewer illegal size lobsters.

Although several types of baits have been tried before (Koyama et al. 1971; Cange et al. 1986; Huner et al. 1990; Miller 1990; Brown et al. 1995; Mohan-Rajan and Shahul 1995), none of them have been successfully used on a commercial scale. This could have been due to different reasons like the cost of those baits, hygienic conditions or space in the fishing boats. This bait presented here could be of importance since uses inexpensive feed ingredients and stores readily without refrigeration; are microbial safe and its size make it easy to handle in a small boat. Nevertheless, in order to make the present baits commercially available, the direct involvement of the Cooperatives will be necessary since they will be the principal or the only buyers of these type of baits since the lobster is on concession to them.

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DESCRIPTIVE STATISTICS OF FISHING PRACTICES, POSTHARVEST HEALTH STATUS, AND TRANSPORT CONDITIONS IN THE PRINCE EDWARD ISLAND LOBSTER (*HOMARUS AMERICANUS*) INDUSTRY

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ABSTRACT This study describes and compares lobster fishing and handling practices on various boats, transportation conditions between fishing wharfs and processing plants, and health assessments of lobsters followed from the time of harvest to the time of arrival at the processing plants during the spring and fall fishing seasons of Prince Edward Island, Canada. A total of 2,191 lobsters landed from 64 boats in 1997 were tagged and included in the study. Over 20 fishing and transport-level factors were monitored, and more than 10 lobster-level factors were assessed on market-sized lobsters. A significant increase ($P < 0.05$) of 7.1% in the proportion of lobsters with open wounds from the time of harvest to the time of entry in the processing plant was found during the spring season; whereas, the proportion of lobsters with vigor loss significantly increased by 2.5% ($P < 0.05$) during the same interval. Total hemocyte counts (THC) and hemolymph total protein (TP) levels were significantly higher in the spring than in the fall ($P < 0.001$). THC and TP also increased significantly ($P < 0.05$) from the time of harvest to the time of arrival at the processing plants, a period in which the lobsters were held out of the water. The prevalence of *Aerococcus viridans* infected lobsters was significantly ($P < 0.001$) higher in the fall season (10.4%) than in the spring season (5.5%). Lobster catches experienced significantly warmer, windier, and sunnier conditions in the fall season ($P < 0.05$). Mackerel was the bait most commonly used during both seasons, and gaspereaux were only used during the fall season. In the spring season, lobsters of different sizes were prevented from having mutual contact on more than 63% of the boats, but only on 18% of the boats in the fall season. Most spring fishers (83.1%) added water to the live-tank after all the traps were hauled, as compared to a majority of fall fishers (72.7%), who had no water in the live-tank at any time ($P < 0.001$). Finally, lobsters spent, on average, significantly ($P < 0.001$) more time on board fishing vessels in the fall than in the spring season.

KEY WORDS: Lobster, *Homarus americanus*, fishing practices, postharvest, health

INTRODUCTION

The lobster (*Homarus americanus*, H. Milne Edwards 1837) fishery is one of the most economically important fisheries in Atlantic Canada. Although Canadian landings reached a record peak in 1991 with more than 48,500 metric tons, they have remained more or less stable since 1992 at about 40,000 metric tons, with an estimated landed value of almost $400 million (Can) in 1997 (Fisheries and Ocean Canada 1999). In 1997, Prince Edward Island fishers landed a total of 8,096 metric tons (20.8% of total Canadian landings) with a value of more than $73.8 million (Fisheries and Ocean Canada 1999).

To supply the market with live product year-round, the industry holds live lobsters in captivity for various periods, ranging from a few hours to several months. However, preprocessing mortalities can cause tremendous losses. These losses have been estimated by the lobster industry to be in the range of 10–15% (Cawthorn 1997). The ability of the industry to maximize economic returns is reduced by lack of knowledge of factors contributing to these losses. Presently, there are no cohesive strategies that link all participants, from fishers to consumers, to detect and quantify production inefficiencies.

The lobster fishery is primarily a specialized in-shore small boat fishery (Pringle and Burke 1993). There is a consensus in the Canadian lobster industry that substantial variation in the quality of the product landed by different fishing boats and also among different fishing wharves is present. Variations in lobster health at the time of arrival at processing plants and storage facilities, and when lobsters are removed from short-term or long-term holding, can be partially attributed to conditions at the time of landing. Therefore, wharf-level factors and boat-level factors would directly influence lobster health. To understand fully the total variation of lobster health that is affected by fishing boats, further assessment at the boat level is needed. Paterson and Spanoghe (1997) suggested that sampling lobsters at various points of handling should yield valid information on stressors causing fatigue, weakness, and death. Following and assessing lobsters through the different handling points enabled the current study to produce a precise estimate of variation in individual lobster health. Identification of the frequency of losses and correlation to specific fishing or handling practices could contribute to better management. Subsequent reduction of losses, even by minimal amounts, could provide significant increases in economic returns to the lobster industry. Fishers, buyers, pound operators, processors, and exporters

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might change their handling methods if provided with results that demonstrate which handling events affect productivity.

The primary objectives were to assess lobster health immediately after removal from the fishing traps, to reassess their health at the different handling points, and to describe fishing practices, handling events, and lobster transportation conditions with possible effects on lobster health in Prince Edward Island during the 1997 spring and fall fishing seasons. A subsequent paper (Lavallée et al. 2000) evaluates relationships between risk factors and lobster health.

MATERIALS AND METHODS

Several lobster health indices were monitored on fishing boats during the 1997 spring (May and June) fishing season in federally designated Lobster Fishing Areas (LFAs) 24 and 26, in Prince Edward Island. Lobster health was also monitored at wharves and processing plants where lobsters were handled. Sampling was repeated during the early part of the 1997 Prince Edward Island fall fishing season in LFA 25 (mid-August to mid-October). A total of 12 wharves in the spring season and three wharves in the fall season were included in the study.

Fishing Boat and Wharf Selection

A list of all lobster fishing wharves located in Prince Edward Island was provided by the Department of Fisheries and Oceans Canada (Charlottetown, PEI). A randomized sample of wharves taken from all lobster fishing wharves with more than 10 boats per wharf was computer generated (Minitab®, version 10.1, Minitab Incorporated, State College, PA, USA, 1994) and divided into two components based upon the fishing season: spring or fall. A brochure describing the project was distributed to fishers at each wharf sampled. Fishers were asked to accommodate an extra person on board to tag and assess lobsters landed on 1 day and also to monitor fishing practices. Fishers were free to decline participation. An average of two wharves were sampled each week, depending on weather conditions and fishers' cooperation. Fishers were selected randomly when more participants than needed agreed to cooperate. Sample-size determinations were based on the maximum expected number of boats on which fishing practices could be evaluated and lobsters tagged and assessed, according to the length of the fishing season (spring) and the availability of technical help (fall).

Lobster Selection

Only market-sized lobsters with a minimum carapace length of 81 millimeters (mm) were included in this study. Individual lobsters were identified by placing a 25.5 cm one-piece prenumbered plastic tag with a pull-tight seal (Ketchum Manufacturing Inc., Ottawa, ON) around the knuckle, proximal to the claw. Whenever sufficient lobsters were available, a sample size of at least 50 lobsters, based on the average number of individual market-sized lobsters expected per boat, was identified using this tagging method.

Physical Examination of Lobsters

Carapace length (measured in mm), sex, shell score (from 0 to 5, depending on the severity of the lesions), and physical condition index were recorded for every tagged lobster. Lobster physical condition index encompassed assessments of damaged or missing claws, legs and antennae, damaged body, open wounds, and vigor status (normal, decreased, or dead). These assessments were performed on the boats immediately after lobsters were removed from the traps. Body weight, recorded in kilograms (Accu-Weigh DSY-1000, Industrial Scales Ltd., Surrey, BC), shell score, and physical condition index were also measured on every tagged lobster at fishing wharves and at arrival at the processing plant, whenever possible.

Hemolymph Sampling

Hemolymph was sampled from a systematic random sample of approximately 15 lobsters per boat for determination of hemolymph total protein (TP), total hemocyte counts (THC), and evidence of *Aerococcus viridans* (causative agent of gaffkemia) infection status. Whenever possible, the same sampling procedures were repeated when tagged lobsters were landed at the wharf and after they reached the processing plant.

Using a 3-mL syringe with a 23-gauge needle, 1.6 mL of hemolymph was aseptically removed from the ventral sinus after swabbing the surface with 70% alcohol. THC were performed according to the method described by Horney et al. (2000): 0.5 mL of hemolymph was added to a 10-mL plastic tube containing 4.5 mL of artificial sea water (ASW) buffer with 0.1% formalin. The tubes were inverted 20–30 times and placed on ice until cell counts were performed with a hemocytometer (double dark line Neubauer improved counting chamber, la Fontaine, Dynatech, Germany) using light microscopy (Zeiss Standard 16 microscope, Germany) with a 40 power objective, by counting 20 squares per sample. The presumptive phenylethylalcohol (PEA) broth test was used for *A. viridans* isolation (Stewart et al. 1966) by adding 0.5 mL of hemolymph to 4.5 mL of PEA broth, vigorously shaking and incubating at 28 °C for 96 h. Duplicate PEA broth cultures were inoculated for each lobster. Suspicious broth culture tubes were identified by the typical purple to green to yellow color change of the broth, and confirmatory testing for the presence of *A. viridans* (tetrad-forming Gram-positive cocci) was performed using Gram stain and microscopic examination. The remaining 0.1 mL of hemolymph was placed on a temperature compensated refractometer for direct reading of total solid protein. The readings were then incorporated in the following formula to be converted to total protein as if measured by the biuret method (Horney et al. 2000):

$$\text{Lobster TP} = 0.898\ (\text{refractometer reading}) - 7.3$$

Assessment of Fishing Practices

A three-part data collection form was used on board each fishing boat from which lobsters were sampled. The first part, *Identification and Boat Specifications*, collected data on the crew size and number of years of experience of the captain or owner of the boat.

The second part, *Environment*, included information on air and surface water (less than 1 meter deep) temperature (°C). Strength (from none to storm), duration (in hours), and description of precipitation (intermittent or continuous) were recorded. In addition, this part included information on sunshine (in hours) and wave conditions (from none to >2 meters). Wind speed was also subjectively scored from 0 to 4 (0 = none, 1 = light, 2 = moderate, 3 = strong, and 4 = storm).

The third part of the data collection form, *Fishing Practices*, collected data on how lobsters were caught and handled on the boats. Bait used in the traps was classified as gaspereaux, mackerel, herring, flatfish, eel pout, redfish, crab, or other. The use of

a protective tarp over the lobster crates or totes, possible contact among lobsters before banding, having the lobsters loose on the deck at any point, and "packing over" and "dumping" of the lobster, either by the fishers or the buyers, were also evaluated. Packing lobsters over was defined as completely emptying a crate by taking each lobster one by one and repacking them into another container. Dumping lobsters was defined as transferring the entire crate or tote into another container simultaneously.

Holding units (small containers in which lobsters are first placed after being removed from the traps and after being graded/banded), and before and after grading were recorded as wooden crate, plastic tote, barrel, tray, polyvinyl chloride (PVC) tube, homemade box, other, or none. The live-tank system (large containers in which the holding units are placed) used on board the fishing vessels was recorded as none, "X-Actics™ box," fiberglass tank, or other. The presence of a lid while fishing and on the way back to the wharf was ranked as either none, partially on, or totally on. In addition, data were collected on the availability of water in the tank system while fishing and on the way back to shore, with the following six categories: "none;" "stagnant," where water was poured on the lobsters but with no flushing; "flow-through," where water was constantly pumped in the live-tank; "poured on," when water was poured on the lobsters and immediately flushed out; "ice," if the lobster were kept on ice; and "other." Furthermore, information was recorded on the maximum and minimum time a lobster could have been on the boat: the maximum time represented the period from when the first lobster was caught until the boat arrived at the wharf, while the minimum time was the same interval for the last lobster caught. The over-all handling of the lobsters on each boat was graded as either generally "placing" or "tossing" the lobster from the traps to the temporary holding units. Finally, the trap setting configuration used by the fishers was documented with four categories: single (one trap per buoy), double (two traps per buoy), multiple (more than two traps on a longline marked by two buoys), or a combination of the previous three methods. The depth (maximal and minimal) at which the traps were set was recorded in meters.

Assessment of Transport Conditions

The different transportation vehicles and conditions were also recorded by the investigators. Data collected included a general description of the vehicle and whether lobsters were transported in an open bed truck (pick-up truck), in a permanently closed transportation compartment (without refrigeration), or in a refrigerated transportation compartment. The outside temperature was recorded in degrees Celsius, and the weather conditions were subjectively described. Availability and of ice was noted as YES or NO, and the type of ice during transport was noted as either, freshwater or saltwater ice. The use of wooden crates or plastic totes was also recorded. Finally, the time interval between the fishing wharf and the processing plant and the total time the shipment spent in the vehicle were recorded in hours.

Statistical Analysis

All data collected were entered in a computer using spreadsheet software (Quattro® Pro version 7, Corel Corporation Limited, Ottawa, Ontario, Canada, 1996). A random sample of 120 records was examined for data entry errors by comparing against original datasheets. The dataset was transferred into a statistical software package (STATA™ 5.0, Stata Corporation, College Station, Texas, USA, 1996) for further analysis. Validation of data was done by obtaining descriptive statistics and looking for outliers. Frequency distributions were generated for each categorical variable and collapsed into dichotomous variables if obvious distribution patterns were seen. Analysis included descriptive statistics, binomial probability tests for gender ratios, Chi-square tests for comparisons of proportions, *t*-tests for comparisons of means in continuous variables, multiple comparisons (analysis of variance; ANOVA) with Bonferroni adjustments for categorical variables. For all analyses, differences were considered significant when $P < 0.05$. Results are presented as mean values ± standard deviation (SD), or proportions with the corresponding binomial exact 95% confidence interval (CI).

RESULTS

Fishing Wharves, Boats, and Lobsters Assessed

The number of tagged and sampled lobsters was limited by the daily catch. In total, 2,191 lobsters (1,672 in the spring and 519 in the fall) from 64 boats (53 in the spring and 11 in the fall) were examined. Approximately 74.5% (1,245 lobsters) of the lobsters tagged on board fishing boats during the spring season were followed to processing plants compared to 61.1% (317 lobsters) in the fall season. Finally, 36.4% (609 lobsters) of the lobsters examined on the boats in the spring had hemolymph sampled; whereas, this proportion was 31.8% (165 lobsters) in the fall. The compliance was excellent in the spring, with only one fisher out of 52 who were approached declining to participate; whereas, the fall compliance was fair with five fishers out of 16 declining to participate in the study.

Physical Examination of Lobsters

The sex ratio of tagged lobsters for the spring and fall seasons together was not significantly different than 1:1, with 52.0% females and 48.0% males ($n = 2,180$ and $P = 0.07$). However, the gender ratios differed significantly from a 1:1 distribution in the spring ($n = 1,665$) and the fall ($n = 708$): 44.5% of the lobsters were males in the spring compared to 59.4% males in the fall.

No significant seasonal differences in lobster body weight ($P = 0.708$) or carapace length ($P = 0.872$) were observed. Lobster body weight for both seasons ranged from 0.264 kg to 2.318 kg with a mean of 0.599 kg (±0.242 kg), and the mean carapace length for both seasons pooled together was 89.1 mm (±9.2 mm) and ranged from 77 mm to 154 mm.

With the exception of vigor, statistically significant differences were observed between the spring and the fall seasons in every physical index assessed, and these differences were present at every source of assessment with a consistent higher proportion of lobsters with normal indices in the fall (Table 1). Although no decrease in the proportion of lobsters with normal physical index was observed in the fall between the time of harvest and the time of landing or the time of arrival at the processing plant, some significant differences were recorded in the spring season. The proportion of lobsters with normal vigor at the processing plant was significantly lower than at the time of harvest or time of landing (Table 1). A significant increase in the proportion of wounded lobsters was also observed between the time of harvest and the arrival at processing plants, as reflected by the decrease in the proportions of normal legs and antennae at the processing

TABLE 1.

Percentage of lobsters with normal physical indices for each Zone, and at each assessment source during the 1997 spring and fall lobster fishing seasons in PEI.

	Lobsters with Normal Indices (%)	
Physical Indices	**Spring Seasnn**	**Fall Season**
Claws		
Boat	$85.3^{a,1}$	$93.1^{b,1}$
Wharf	$83.5^{a,1}$	$93.8^{b,1}$
Processing plant	$83.7^{a,1}$	$90.2^{b,1}$
Legs		
Boat	$93.7^{a,1}$	$98.1^{b,1}$
Wharf	$88.7^{a,2}$	$97.9^{b,1}$
Processing plant	$88.6^{a,2}$	$96.9^{b,1}$
Antennae		
Boat	$86.8^{a,1}$	$98.7^{b,1}$
Wharf	$85.7^{a,1,2}$	$97.9^{b,1}$
Processing plant	$82.8^{a,2}$	$97.8^{b,1}$
Body		
Boat	$93.1^{a,1}$	$96.9^{b,1}$
Wharf	$88.5^{a,2}$	$97.2^{b,1}$
Processing plant	$90.6^{a,1,2}$	$96.5^{b,1}$
Wound		
Boat	$87.9^{a,1}$	$94.8^{b,1}$
Wharf	$84.1^{a,1,2}$	$93.1^{b,1}$
Processing plant	$80.8^{a,2}$	$92.1^{b,1}$
Vigor		
Boat	$99.7^{a,1}$	$98.1^{a,1}$
Wharf	$99.1^{a,1}$	$98.6^{a,1}$
Processing plant	$97.2^{a,2}$	$96.9^{a,1}$

Significant differences are represented by different superscripts: letters within each row and numbers within each column. The normal score for the index "wounds" indicates the absence of wounds. In the spring, n = 1,672 on the boats, 866 at the wharves and 1,245 at the processing plants, and in the fall, n = 516 on the boats, 145 at the wharves and 317 at the processing plants.

plants. None of the lobsters assessed during this study suffered from shell disease.

Hematology

Hemolymph Total Protein (TP)

For every source of assessment (boat, wharf, and processing plant), the mean TP levels in the spring season were significantly higher than TP levels in the fall season for both male and female lobsters, with mean values ranging between 57.6 g/L and 70.5 g/L in the spring and between 31.9 g/L and 47.5 g/L in the fall (Table 2). Female lobsters had significantly higher TP levels than male lobsters at ever assessment source in the spring and also at the processing plant in the fall season, but male lobsters had higher TP levels at the wharf than females in the fall (Table 2). In the spring season, TP values of female lobsters were significantly higher at the time of landing than at the time of harvest (Table 2).

Total Hemocyte Counts (THC)

Significantly lower THC values were observed at the boat level during the spring season, as compared to the fall season; whereas, higher counts were seen at the time of landing in the spring, as compared to the fall season (Table 2). Similar to TP, some gender differences in THC were also observed in both fishing seasons: in the spring, the only significant difference between sexes was observed at the time of landing, with male lobsters having a mean THC lower than female lobsters. In the fall season, male lobsters also had lower THC than female lobsters at the time of arrival at processing plants, but had significantly higher THC than females at the time of harvest (Table 2). For both male and female lobsters during the spring season, significant increases in THC were observed between the time of harvest and the time of landing. Increases in females THC in the spring and fall were also significant between the time of harvest and the arrival at processing plants, while being significant only in the spring for male lobsters (Table 2).

Aerococcus Viridans Prevalence

The spring prevalence (n = 635) of *Aerococcus viridans*, the causative agent of gaffkemia was estimated at 5.33% (3.88%, 7.61%) and was significantly lower ($P < 0.001$) than the fall prevalence (n = 173) of 10.41% (6.28%, 15.95%). No significant difference in the prevalence of *A. viridans* according to sex in either season was observed.

Fishing Practices

Crew Size and Experience

There was no significant difference in the distribution of the size of the crew between the spring and fall seasons, and overall, the majority of crews consisted of fewer than three persons (Table 3). A significant difference between the two seasons ($P = 0.037$) was observed in the mean number of years of experience of the captain with fall captains being more experienced (Table 3).

Environmental Factors

The maximum and minimum air temperatures and the water temperature were all significantly lower ($P < 0.001$) in the spring than in the fall (Table 3). A significant difference ($P = 0.024$) was also found in the amount of sunshine during fishing, between the spring and fall seasons, but not in rainfall (Table 3). The amount of sunshine was greater than 6 hours on a majority of boats in the fall; whereas, the majority of spring boats experienced 6 hours or less. No significant difference was observed in the wave conditions or the wind velocity between season, and overall, the waves were estimated to be of an approximate height of one meter or less; whereas, on a majority of boats, the wind was scored as being none to light (Table 3).

Setting Configuratinn of Traps and Bait

Fishers used four different trap setting methods: single, double, multiple, or a combination of the previous three methods. A significant difference ($P < 0.001$) in the methods used was found between seasons (Table 3). For example, multiple traps per line were used on 95.6% of the boats in the spring, but never used in the fall fishing season. Significant differences were present between seasons ($P = 0.005$ for the minimum depth and $P < 0.001$ for the maximum depth), and both the maximum and minimum depths at which traps were set during the fall were greater than during the spring (Table 3). A significant difference ($P < 0.001$) was observed in the distribution of baits used between the two fishing seasons; although mackerel was the bait most commonly used in both seasons, gaspereaux was only used in the spring, and

TABLE 2.

Range, mean, and standard deviation of total hemolymph protein (TP) and total hemocyte counts (THC), according to the source of assessment and the fishing zone during the 1997 spring and fall lobster fishing seasons in PEI.

Variable	Source	Spring		Fall	
		Gender	Mean (SD)	Gender	Mean (SD)
Total protein (g/L)	Boat	Male	57.6 (14.3)^{a,1,†}	Male	39.8 (12.3)^{a,2,†}
		Female	65.4 (21.9)^{a,1,‡}	Female	40.5 (22.7)^{a,2,†}
	Wharf	Male	60.4 (12.5)^{a,1,†}	Male	38.9 (7.9)^{a,2,†}
		Female	70.5 (20.0)^{b,1,‡}	Female	31.9 (11.5)^{a,2,‡}
	Processing plant	Male	59.5 (12.4)^{a,1,†}	Male	40.7 (10.8)^{a,2,†}
		Female	65.6 (20.8)^{a,1,‡}	Female	47.5 (22.9)^{a,2,‡}
Hemocyte counts ($\times 10^6$ cells/ml)	Boat	Male	20.4 (8.5)^{a,1,†}	Male	25.3 (7.9)^{a,2,†}
		Female	20.8 (10.7)^{a,1,†}	Female	20.6 (7.3)^{a,1,‡}
	Wharf	Male	30.2 (9.2)^{b,1,†}	Male	27.8 (8.2)^{a,1,†}
		Female	33.4 (11.3)^{b,1,‡}	Female	26.9 (3.7)^{a,b,1,†}
	Processing plant	Male	30.3 (10.3)^{b,1,†}	Male	27.5 (8.6)^{a,1,†}
		Female	32.0 (15.8)^{b,1,†}	Female	31.3 (13.8)^{b,1,†}

Significant differences are represented by different superscripts: letters for the differences among sources of assessment for each both variables, in each gender and each season; numbers for the differences between season for both variables, in every source and each gender; and symbols († and ‡) for differences between gender for both variables, in every source and each season.

herring was used more often in the fall than in the spring lobster fishing season (Table 3).

Contact Before Banding and Over-all Handling of Lobsters

The proportion of boats on which physical contact among lobsters was prevented until they were measured and had their claws banded was significantly higher ($P < 0.001$) in the spring than in the fall (Table 3). Market-sized lobsters, after being removed from the traps, were generally tossed rather than placed into the temporary holding units on only 25% of the boats in total, and no difference was observed between seasons (Table 3).

Holding Unit and Live-Tank System

In both fishing seasons, the traditional plastic tote with a storage capacity of approximately 35 kg, represented the holding unit mostly used onboard fishing boats, whether it was before or after the lobsters were measured, graded, and banded (Table 3). Although no significant difference was noted between seasons in the distribution of the holding units used after grading, a difference ($P < 0.001$) was observed before grading; during the spring, the wooden crate was not used, as compared to more than 45% for the fall fishers. No difference between seasons was observed in the type of live-tank used on board the fishing boats, and overall, the fiberglass tank was mostly used (Table 3).

Water Availability and Lid Cover with the Live-Tank

In the spring, a majority of the fishers waited until all traps had been retrieved before adding stagnant water into the live-tank, and this practice was significantly different ($P < 0.001$) than during the fall season during which, most fishers never added water into the live-tank (Table 3). However, most fall fishers had the lid cover completely on the live-tank at any time; whereas, only 38% of the spring fishers had the cover completely on during fishing (Table 3). The difference between season in the lid availability distribution was only significant during fishing ($P < 0.001$), and not once all the traps had been retrieved.

Time on Board Fishing Boats

Only the maximum amount of time lobsters spent on board fishing boats was significantly different between the spring and the fall seasons ($P < 0.001$), with longer maximum time on board fishing vessels in the fall season (Table 3).

Transport Conditions

Transportation Vehicles and Ice with Transportation

A significant difference ($P < 0.001$) was present in the different vehicles used between spring and fall seasons to transport lobsters from wharves to processing plants (Table 4). During the spring, the vehicles mostly used consisted of trucks with refrigerated transportation compartments; whereas, in the fall, only closed trucks without refrigeration were used. Whenever ice was used around lobster crates or totes, it was freshwater ice, and during both seasons, a majority of shipments were sent to processing plants without ice.

Transportation Interval—Traveling and Shipping Intervals

The traveling and the shipping intervals showed significant differences ($P < 0.001$ for both intervals) between seasons, being shorter in the fall season than in the spring season (Table 4).

Air Temperature and Wind Velocity During Road Transport

The average temperature during transportation of lobsters from the wharf to the processing plant was significantly ($P < 0.001$) lower in the spring than in the fall (Table 4). It was also significantly ($P = 0.037$) windier in the fall fishing season than in the spring (Table 4).

DISCUSSION

The over-all compliance from the fishing and processing sectors of the lobster industry during this study was good, suggesting increasing concerns from the industry in lobster health-related issues. Although only market-sized lobsters were to be included in

TABLE 3.

Fishing factors assessed during the 1997 spring and fall lobster fishing season in PEI, with corresponding distribution (proportion) for each category or corresponding mean (SD).

Fishing Factor	Fishing Season		
	Spring	Fall	Overall
Crew size			
Less than 3 persons	26 (57.8%)	5 (45.5%)	31 (55.4%)
3 persons or more	19 (42.2%)	6 (55.4)	25 (44.6%)
Captain's experience, in years	18.2 (11.0)	27.1 (13.0)	19.1 (10.9)
Maximum air temperature, in °C	15.4 (4.0)	21.8 (4.8)	16.5 (5.1)
Minimum air temperature, in °C	8.2 (3.8)	13.7 (1.7)	9.3 (4.2)
Surface water temperature, in °C	7.9 (3.2)	17.2 (1.2)	9.1 (4.8)
Rain			
None	33 (73.4%)	7 (63.6%)	40 (61.4%)
Light to moderate	12 (26.6%)	4 (36.4%)	16 (38.6%)
Sun			
None	11 (24.4%)	2 (18.2%)	13 (23.2%)
6 hours or less	29 (64.5%)	3 (27.3%)	32 (41.1%)
More than 6 hours	5 (11.1%)	6 (54.5%)	11 (19.7%)
Wave			
1 meter or less	36 (80.0%)	6 (54.5%)	42 (75.0%)
More than 1 meter	9 (20.0%)	5 (45.5%)	14 (25.0%)
Wind			
None to light	33 (73.4%)	7 (63.6%)	40 (61.4%)
Moderate to strong	12 (26.6%)	4 (36.4%)	16 (38.6%)
Trap setting method			
Single or double	1 (2.2%)	7 (63.6%)	8 (14.3%)
Multiple	43 (95.6%)	0 (0.0%)	43 (76.8%)
Combination	1 (2.2%)	4 (36.4%)	5 (8.9%)
Max. trap setting depth, in m	17.7 (6.0)	28.0 (3.1)	19.3 (6.8)
Min. trap setting depth, in m	6.0 (4.3)	12.0 (7.6)	6.9 (5.3)
Bait			
Gaspereaux	11 (24.1%)	0 (0.0%)	11 (14.9%)
Mackerel	19 (41.4%)	13 (46.4%)	32 (43.2%)
Herring	4 (8.7%)	7 (25.0%)	11 (14.9%)
Flatfish	5 (10.3%)	1 (3.6%)	6 (8.1%)
Other	7 (16.1%)	7 (25.0%)	14 (18.9%)
Contact before handing			
Yes	14 (34.1%)	9 (81.8%)	23 (44.2%)
No	27 (65.9%)	2 (18.2%)	29 (55.8%)
Lobster handling			
Placed	30 (73.2%)	9 (81.8%)	39 (75.0%)
Tossed	11 (26.8%)	2 (18.2%)	13 (25.0%)
Holding unit before grading			
Tote	37 (69.8%)	6 (54.5%)	43 (67.2%)
Crate	0 (0.0%)	5 (45.5%)	5 (7.8%)
Other	7 (13.2%)	0 (0.0%)	7 (10.9%)
None	9 (17.0%)	0 (0.0%)	9 (14.1%)
Holding unit after grading			
Tote	51 (96.2%)	9 (81.8%)	60 (93.8%)
Crate	2 (3.8%)	2 (18.2%)	4 (6.2%)
Live-tank			
Fiberglass	32 (71.1%)	4 (36.4%)	36 (64.3%)
X-Actics™	10 (22.2%)	5 (45.4%)	15 (26.8%)
Other	3 (6.7%)	2 (18.2%)	5 (8.9%)
Water availability during fishing			
Stagnant	19 (35.9%)	1 (9.1%)	20 (31.3%)
Other	6 (11.3%)	2 (18.2%)	8 (12.5%)
None	28 (52.8%)	8 (72.7%)	36 (56.2%)
Water availability after fishing			
Stagnant	44 (83.0%)	1 (9.1%)	45 (70.3%)
Other	2 (3.8%)	1 (9.1%)	3 (4.7%)
None	7 (13.2%)	9 (81.8%)	16 (25.0%)
Lid on live-tank during fishing			
Completely on	17 (37.8%)	10 (90.9%)	27 (48.2%)
Partially on	18 (40.0%)	0 (0.0%)	18 (32.1%)
None	10 (22.2%)	1 (9.1%)	11 (19.7%)
Lid on live-tank after fishing			
Completely on	28 (62.2%)	10 (90.9%)	38 (67.9%)
Partially on	13 (28.9%)	0 (0.0%)	13 (23.2%)
None	4 (8.9%)	1 (9.1%)	5 (8.9%)
Maximum time on board[1]			
Between 4 and 6 hours	28 (62.2%)	3 (27.3%)	31 (55.4%)
6 hours or more	17 (37.8%)	8 (72.7%)	25 (44.6%)
Maximum time on board[2]			
2 hours or less	44 (97.8%)	10 (90.9%)	54 (96.4%)
More than 2 hours	1 (2.2%)	1 (9.1%)	2 (3.6%)

[1] The maximum time represented the period from when the first lobster was caught until the boat arrived at the wharf.

[2] The minimum time represented the period from when the last lobster was caught until the boat arrived at the wharf.

this study, one canner-sized lobster (carapace length < 81 mm) was tagged and probably represents one canner-sized lobster that was misplaced by the fisher with the market-sized lobsters. Only the handling and fishing practices that showed substantial results are discussed. These include the lobster-level factors of gender, physiological indicators of health and vigor, environmental conditions, and boat-level factors related to traps and bait, holding and live-tank systems, and, finally, the over-all lobster handling. All remaining factors, including all transportation condition factors, did not show important variations and are not discussed further. The uneven distribution of wharves between the spring and the fall seasons was attributable to the limited time the investigators had to carry the study into the fall season. Because of the variation in sample sizes in the different fishing seasons, caution must be taken when comparing results from these lobster fishing seasons.

Physical Examination of Lobsters

Lobster Physical Conditinn and Vigor

The initial assumption was that lobster health status would being to decrease only after lobsters entered the traps. Most of the vigor loss occurred between wharves and processing plants, not between boats and wharves. Perhaps these vigor losses were induced by injuries inflicted on the wharves or even on the boats, but their effect was not detected until later. The fall fishing season is occasionally considered a lesser quality fishery because a majority of lobsters are in a postmolting softer-shelled condition. Therefore, the significant higher proportion of lobsters with normal physical

TABLE 4.

Transportation factors assessed during the 1997 spring and fall lobster fishing seasons in PEI, with corresponding distribution (proportion) for each category, or corresponding mean (SD).

Transport Factor	Fishing Season		
	Spring	Fall	Overall
Vehicle			
Direct[1]	7 (17.5%)	0 (0.0%)	7 (14.3%)
Refrigerated truck	26 (65.0%)	0 (0.0%)	26 (53.1%)
Nonrefrigerated truck	4 (10.0%)	9 (100.0%)	13 (26.5%)
Unknown	3 (7.5%)	0 (0.0%)	3 (6.1%)
Road shipment on ice			
Yes	8 (25.0%)	3 (33.3%)	11 (26.8%)
No	24 (75.0%)	6 (66.7%)	30 (73.2%)
Travelling interval			
Direct[1]	7 (17.5%)	0 (0.0%)	7 (14.3%)
1 hour or less	15 (37.5%)	9 (100.0%)	24 (49.0%)
Between 1 and 4 hours	15 (37.5%)	0 (0.0%)	15 (30.6%)
4 hours or more	3 (7.5%)	0 (0.0%)	3 (6.1%)
Shipping interval			
Direct[1]	7 (17.5%)	0 (0.0%)	7 (14.3%)
1 hour or less	6 (15.0%)	6 (66.7%)	12 (24.5%)
Between 1 and 4 hours	13 (32.5%)	3 (33.3%)	16 (32.6%)
4 hours or more	14 (35.0%)	0 (0.0%)	14 (28.6%)
Outside air temperature, in °C	15.3 (6.6)	20.6 (1.1)	16.1 (6.4)
Wind during transport			
Light	32 (88.9%)	4 (57.1%)	36 (83.7%)
Moderate	4 (11.1%)	3 (42.9%)	7 (16.3%)

[1] Direct meant that no vehicle was used; the lobsters were landed directly at the processing plants.

indices in the fall compared to the spring season was unexpected, and the explanation is unclear.

Gender

Ennis (1978) found that the male–female ratio of large lobsters when sampled by scuba divers was 1:1, but when estimated through trap capture, the ratio was 3:1 in favor of male lobsters. The over-all sex ratio of all tagged lobsters included in this study was 52% females versus 48% males, but significantly more female lobsters were observed in the spring season than in the fall season. Assuming a normal distribution of the sex ratio among all hatched lobster eggs, and equal survival rates among female and male lobsters to market size, a 50% male, 50% female population should be available for harvest. Federal regulations require that all ovigerous females must be returned to the water, and the removal of their eggs is prohibited (Miller 1995). By protecting ovigerous females, fewer females should be landed, because on average, female lobsters will carry eggs externally for almost a year (Waddy et al. 1995), thus increasing the likelihood of catching more males at any point in time. The reason for the higher proportion of females landed in the spring season is still unclear, but could reflect a competitive behavior among males, as suggested by Campbell (1986), or perhaps a difference in the feeding behavior of male versus female lobsters.

In the early part of the Prince Edward Island fall season, a significant proportion of lobsters are in postecdysis. Because female lobsters mate shortly after the ecdysis (Talbot and Helluy 1995); a higher proportion of males caught in this period could reflect shelter-restricted behavior of females who recently molted and mated. Waddy and Aiken (1990) reported a higher relative activity of mature male lobsters than females. These behavioral differences could explain the gender ratio of landed lobsters favoring males over females observed during the fall season.

Hematology

Some hemolymph parameters have been used to define lobster health (Horney et al. 2000, Jussila et al. 1997). The range for THC and TP reported here were very large, mostly attributable to the sampling done during two different fishing seasons. In this study, 95% of the lobster population had TP levels measured on the boats were between 18.6 g/L and 99.8 g/L. The levels of TP reported by Horney et al. (2000) were within the range of these data, with mean values of 19 to 65 g/L, and the values reported by Chen and Chia (1997) in the mud crab (*Scylla serrata*) were also similar, at 81.0–88.4 g/L. Because many external factors can affect the TP and THC levels postlanding, the values obtained when lobsters were taken onto the boats were probably the most representative assessments of the natural situation. Horney et al. (2000) reported mean values for THC in their laboratory study of 6.6 to 31.1 $\times 10^6$ hemocytes/mL, while that reported by Cornick and Stewart (1978) was 18.1 $\times 10^6$ hemocytes/mL. These values are also within the mean THC ± 2 SD (95% of the population) reported in this study (5.0 to 50.5 $\times 10^6$ hemocytes/mL). Jussila et al. (1997) reported a range for THC in western rock lobster (*Panulirus cygnus*) of 2.5 to 15.9 $\times 10^6$ hemocytes/mL, with the highest mean THC in lobsters freshly arrived at the factory tanks, and suggested stress from handling or exposure to air as causative factors for the high mean of THC.

Lobster TP levels are influenced by the time of year, the molting cycle, the water temperature and probably by many other factors including diet, size, and gender (Chen and Chia 1997, Ennis 1973, Horney et al. 2000; Paterson and Spanoghe 1997). Immediately following the completion of the ecdysis, lobsters will ingest and absorb substantial volumes of seawater to expand their volume to often 50% greater than they were before the ecdysis (Aiken and Waddy 1992). This would dilute the hemolymph and result in lower TP levels and lower THC. The fall fishing season of Prince Edward Island is timed to occur after most newly molted lobsters have achieved stage C of the molting cycle, and, therefore, lower TP in the fall were expected. Chen and Chia (1997) reported the lowest protein levels for the mud crab (*Scylla serrata*) during stage B, and the highest levels during stages D_2 and D_3. High level of feeding activities could be necessary to enable lobsters to recover from the immediate low postmolt condition, as suggested by varying serum protein in field-captured lobsters (Ennis 1973).

Female lobsters had significantly higher TP levels than males at every assessed handling point of the industry during the spring season: boat, wharf, and arrival to the processing plant. Horney et al. (2000) only reported a minimal influence of sex on the different components measured in lobster hemolymph held under laboratory conditions. Chen and Chia (1997) reported no significant difference in TP levels between male and female mud crabs (*Scylla serrata*). During the fall, female lobsters had significantly higher TP levels than males only when assessed at entry to processing plants.

Another important finding was the apparent dehydration observed in lobsters kept out of the water, demonstrated by increas-

ing THC, and by TP levels to a lesser degree, from boats to wharves, and additionally from boats to arrival at processing plants. Perhaps lobsters kept out of the water for extended periods are losing considerable amounts of body fluids, resulting in hemoconcentration, and higher THC and TP values. Recently, Jussila et al. (1999) reported that only emersion periods greater than 2 hours induced significant changes in western rock lobster (*Panulirus cygnus*) THC. This correlates with the present findings, because the average emersion period calculated from the time of landing to the time of arrival at processing plants was approximately 3 hours and 15 minutes in the spring. In the fall season, this period averaged only 1 hour and 10 minutes, but because almost 82% of the fishers did not add any water at all in the live-tank after fishing, the total mean period for lobsters were kept out of the water is estimated to be at least 2 hours and 20 minutes. Dehydration has been demonstrated in prawns (*Penaeus japonicus*) to be up to 0.75% loss of body weight per hour of exposure to air, at 75–85% relative humidity (Samet et al. 1996). Newsom et al. (1994) suggested that spraying red swamp crawfish (*Procambarus clarkii*) with seawater could probably protect them against dehydration through evaporation, and may also help replace some of the body fluids lost. Jussila et al. (1999) also suggested that regular handling of lobster (*Panulirus cygnus*) as it happens during postharvest manipulation can elevate THC, and when sampling is performed during handling, especially if delays in sampling are occurring, significant elevation in THC may result after only a few minutes. It is likely that the combination of stress inflicted through increasing handling procedures from harvest time through to arrival at processing plants, and the extensive emersion periods explains the elevated THC observed at the time of arrival at the plant compared to THC estimated directly on fishing boats.

During the spring season, a significant gender difference in the THC values was seen only at the wharves, with females having higher counts. For the fall season, significant differences in THC between male and female lobsters were observed on the boats and at the time of entry into processing plants; females having higher counts than males at the plants, but lower at the boats. Overall, no consistent patterns in the THC according to sex were recorded, similar to the findings of Jussila et al. (1997) on rock lobsters (*Panulirus cygnus*). Cornick and Stewart (1978) did not assess sex differences in THC, although they looked at differential hemocyte counts and reported no significant difference according to gender. Female lobsters do not molt as often as males of comparable sizes because of the reproductive cycle, some variation between gender in both TP and THC values could be present before, during, and shortly after ecdysis, which could explain some of the results of this study.

It may be more appropriate to report TP or THC range instead of mean for indicating or predicting lobster health at the population level. The wide range of TP and THC values observed may be helpful, if certain factors affecting these ranges can be identified, and, therefore, used as indicators of health. Further studies of factors influencing the range instead of the mean values of either TP or THC are required if these factors can first be identified.

The significantly higher prevalence of *Aerococcus viridans* in lobsters from the fall fishing season compared to the spring season is likely a reflection of the warmer water temperatures in the late summer and fall. The only factor that seems to affect the prevalence of clinical disease in *A. viridans* infected lobsters is the water temperature. A decreased mean time to death with increasing water temperatures would result in increased clinical disease incidence and mortality (Bayer and Daniel 1987, Stewart 1975). Therefore, it may be possible that at higher water temperatures, more infective particles are released in the environment via decomposition of dead infected lobsters, thus increasing the likelihood of capturing live infected lobsters. Gaffkemia is a disease endemic to lobster populations of North America, and has also been reported in European waters (Alderman 1996). Huang and Bayer (1989) reported *A. viridans* prevalence of 6.7% in freshly caught lobsters off the coast of Maine. The prevalence of *A. viridans* in Atlantic Canada was estimated at almost 5% by Stewart et al. (1996), while site specific levels ranged from 0 to 22% according to Vachon et al. (1981). Keith et al. (1992) reported that 0 to 40% of lobsters caught in Canadian locations were infected with *A. viridans*. Lobsters cannot control or efficiently respond to infection with *A. viridans* (Marks et al. 1992, Stewart and Zwicker 1974). In *A. viridans* infected lobsters, there is no agglutination and the bactericidal ability of the hemolymph deteriorates in the presence of virulent strains of *A. viridans* (Johnson et al. 1981, Stewart 1975). Lobsters infected with *A. viridans* will eventually die of gaffkemia, which explains some of the confusion in the literature when reporting prevalence of *A. viridans* versus prevalence, or incidence, of gaffkemia in wild lobsters. Nevertheless, these reported infection levels agree with the prevalence estimated in this study that was 5.5% in the spring and 10.4% in the fall.

Environmental Factors

Lobsters are sensitive to freshwater exposure (Ennis 1995, Jury et al. 1994, McMahon 1995), and therefore, heavy exposure to rain will likely be detrimental to lobsters, especially for prolonged exposure times. Furthermore, exposure to sunshine could result in more rapid drying of the external surface of the lobsters, resulting in a loss of body fluids. Newsom et al. (1994) suggested that water is lost more rapidly than other components of the hemolymph, and that the hemolymph concentration should increase.

Fishing Practices

Stress from harvesting and handling of western rock lobsters has been blamed for productivity losses in the lobster (*Panulirus cygnus*) industry of Western Australia (Paterson and Spanoghe 1997). Similarities with the Canadian lobster fishery are most likely, and the various fishing practices should also have profound impacts on further industry losses. Therefore, a higher proportion of lobster injuries on boats where fishers were less careful with their catch was expected.

Setting of Traps and Bait

Cooler water temperatures at greater depths might have different impacts on lobster health than warmer waters from shallower bays. Greater changes in water temperature and hydrostatic pressure are experienced by lobsters from deeper traps when brought to the surface. Perhaps this change in pressure removes some of the oxygen from the hemocyanin, and could result in anoxia.

The type of bait, its composition, and its quality may physiologically affect lobsters postcapture. During the fall season, most fishers used two or three different types of bait, explaining why the total number of bait-types used exceeded the total number of boats (28 bait-types used by 11 boats). Bait quantity and quality has been reported to influence trap catch yields in crawfish (Romaire 1995). However, Addison and Bell (1997) reported that reduced bait attractiveness had less influence on catch rates than behavioral interactions. Unfortunately, correlations between types of bait used and numbers of lobsters caught were not investigated in this study.

Different trap attraction rates were evaluated when mussels, sea urchins, cattle hocks, animal guts, and diesel oil were used as bait in spiny lobster (*Panulirus* sp.) traps, with mussels and diesel oil being, respectively, the most effective baits (Mohan Rajan et al. 1995). Mussels as bait could represent an interesting alternative in Prince Edward Island, with the important number of mussel farms and processing plants; mussels being culled at harvest could be used in the lobster fishery. Crawfish fishers typically use "fish bait" in cold waters (< 20 °C), but when fishing in warmer waters (< 20 °C), they either use "formulated bait" or a combination of fish and formulated baits (Romaire 1995), and, therefore, a formulated bait could also represent another alternative for the fall lobster fishery.

Holding Unit and Live-Tank System

The tolerance of aquatic crustaceans to exposure to air is greatly increased by cooling the animals, and by having a high relative humidity (Samet and Nakamura 1997). Having ice in the live tank can be an advantageous alternative when no water is used. However, if the water stays stagnant for long periods, oxygen is depleted, and lobsters become hypoxic. Lobsters usually survive for longer periods if they are kept out of water, in a cool environment where their gills stay wet, compared to maintenance in stagnant water with no aeration. Keeping aquatic crustaceans dry is harmful, especially if they are exposed to wind or direct sunshine, and their gills could eventually collapse if the relative humidity drops beyond a critical value for an extended period (Samet and Nakamura 1997). The use of a lid offers some protection from direct sunlight or rain, and can help maintain the relative humidity high and constant in the live tank. The lid may also help in reducing the temperature variation inside the live-tank. Sudden and drastic changes in water temperature are stressful to lobsters, and can strongly influence their behavior (Crossin et al. 1998), and most likely their health status as well. Unfortunately, the water quality of the live-tanks was not assessed during this study and its direct effect could not be documented.

CONCLUSIONS

By tagging and following individual lobsters during the Prince Edward Island spring and fall season, lobster health was assessed at the different handling points of the fishing industry. THC and TP showed increasing levels from the time of harvest of the time of landing and subsequently, to the time of arrival at processing plants, suggesting a possible influence of handling related stress, dehydration, or possible a combination of the two. Fishing practices handling events, and lobster transportation conditions were thoroughly described. Some environmental conditions and fishing practices showed substantial variation between the spring and fall fishing seasons. Fishing conditions were warmer, sunnier, the waves were greater, and traps were set in deeper water in the fall. While fishers from both season used mainly mackerel as bait, gasperaux was only used in the spring season. In the spring season, lobsters of different sizes were prevented from having mutual contact on most boats, but only on a small proportion of boats in the fall season. Most spring fishers waited after all the traps were hauled before adding water to the live-tank compared to a majority of fall fishers who had no water in the live-tank at any time. Finally, lobsters spent on average significantly more time on board fishing vessels in the fall than in the spring season.

Attempting to follow lobsters from fishing boats through wharves and processing plants is a logistical challenge for evaluation of lobster health or productivity. In many field studies, these logistical problems have limited the ability to obtain valid results. This study enabled the investigators to identify the frequency with which a series of different fishing practices, and transportation conditions was used. Paterson (1999) suggested that analytical models could be used to predict certain outcomes, such as lobster weakness state or death from varying lobster stress indicators. By using epidemiological methods where lobster-level factors (stress indicators) and group-level factors (wharf or boat-level), a model predicting associations between factors and lobster survival would be advantageous. In a subsequent paper (Lavallée et al. 2000), the results from this study were used to build an epidemiological model assessing relationships between fishing and handling risk factors for decreased lobster vigor upon arrival at processing plants.

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ANALYTICAL ASSESSMENT OF HANDLING, FISHING PRACTICES, AND TRANSPORTATION RISK FACTORS ON LOBSTER (*HOMARUS AMERICANUS*) HEALTH IN PRINCE EDWARD ISLAND, CANADA

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ABSTRACT The objective of this study was to identify risk factors for productivity losses in the Canadian lobster industry. Lobster handling and fishing practices onboard 64 fishing boats and transportation conditions on 49 vehicles subsequently transporting these lobsters from fishing wharves to processing plants were assessed during the spring and fall fishing seasons of 1997 on Prince Edward Island. These practices and conditions were then related to the health of the lobsters, estimated by the vigor status on arrival at the processing plants. A generalized estimating equation logistic regression model was used to assess the impact of handling, fishing, and transportation practices on lobster vigor. Significant risk factors for loss of vigor included the following boat-level factors: the use of mackerel for bait (odds ratio, OR, of 7.1; $P = 0.003$), tossing (as opposed to placing) lobsters from traps to temporary holding units on board the fishing boats (OR = 3.6, $P = 0.048$), and exposure to rain while on board fishing boats (OR = 3.6, $P = 0.011$), while greater maximal depths at which the traps were set had a protective effect on lobster vigor (OR = 0.85/m, $P = 0.010$).

KEY WORDS: Lobster, *Homarus americanus*, risk factors, postharvest, health

INTRODUCTION

Among Canadian fisheries, the lobster (*Homarus americanus*, H. Milne Edwards 1837) fishery is one of the most important, both in volume and in landed value. It consists primarily of a specialized inshore small boats fishery (Pringle and Burke 1993). Paterson and Spanoghe (1997) suggested that sampling lobsters at various points of handling should yield information on stressors influencing lobster health. The traditional lobster industry in Atlantic Canada is usually represented by the fishing sector that sells its daily catch to buyers located directly at the fishing ports, and these buyers can either sell their live product to processing plants or to seafood companies specializing in live holding. Lobsters can then be shipped live or processed before being exported. Variations in lobster health, before and after short or long-term holding, are hypothesized to be associated with conditions experienced while on fishing vessels and perhaps also during wharf-level handling events.

Several potential risk factors for productivity losses in the Canadian lobster industry were identified by monitoring handling and fishing practices on board fishing boats and during subsequent transport from fishing wharves to processing plants during the 1997 spring and fall fishing seasons in Prince Edward Island (Lavallée et al. 2000). These factors included some crew and boat specifications, such as crew size and years of experience of the captain. They also included the environmental variables rain, sunlight, wind, wave, water, and air temperatures. Fishing practice risk factors investigated included trap setting configuration and depth at which traps were set, lobster handling methods, type of bait, contact among lobsters before grading and banding, type of temporary storage unit and holding tank, water and lid availability for holding tanks, methods of transferring lobsters, and maximum and minimum periods spent on boats. Transportation conditions included the following risk factors: type of vehicle, presence of ice during transportation, type of shipping unit, outside air temperature, and other weather conditions, time interval between wharves and processing plants, and maximum duration shipments stayed in transport vehicles.

The objective of this study was to develop an epidemiological model to assess the impact of different fishing and handling practices previously identified as potential determinants of the health and quality of lobsters upon arrival at processing plants.

MATERIALS AND METHODS

Data Collection and Variable Selection

Data on lobster physical and physiological status, and on fishing practices, handling practices, and transportation conditions that may have a significant impact on lobster health before holding, after holding, or prior to processing and marketing were collected between May 1997 to August 1997 in Prince Edward Island, Canada. Date were obtained by monitoring fishing practices on boats and subsequent transportation conditions as lobsters were moved to processing plants. Fishing and handling practices were monitored daily in 64 groups of lobster. Each group consisted of all market-sized lobsters (carapace length ≥81 mm) caught on one boat during one day. These groups were subsequently transferred to processing plants by 49 different vehicles. Lobster health was assessed directly on board the fishing vessels, and later when

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groups were landed, and eventually at the processing plants (see Lavallée et al. 2000 for further description of the data collection process).

Variables recorded were a mixture of continuous, ordinal, nominal, and dichotomous variables. Continuous variables were converted to dichotomous variables with two levels when the independent variable showed a tendency to group around discrete values. The outcome variable for the model was a dichotomous variable describing the vigor of each lobster upon arrival at processing plants. Every lobster was individually observed, and a lobster showing any delay or absence of response (i.e., tail flipping, claw(s) rising, antenna(e) movement) to physical stimuli was given a score of "1" (decreased vigor); whereas, a lobster showing immediate response to physical stimuli was given a score of "0" (normal vigor).

Statistical Analysis

The dataset was transferred into a statistical software package (STATA™ version 5.0, Stata Corporation, College Station, Texas, USA, 1996). After transformation of all categorical variables into dichotomous variables, 47 explanatory variables were retained. Unconditional associations between the outcome (lobster vigor score at arrival at the processing plant) and the predictors were evaluated by Chi-square tests for dichotomous variables (with corresponding relative risks) and by *t*-tests for the continuous variables. For all analyses, observed associations were considered significant when $P < 0.05$.

Variables with significant unconditional associations with the dependent variable (lobster vigor) were selected for inclusion in a multiple variable model building process. The model used was a logistic regression model using a generalized estimating equation (GEE) procedure (Liang and Zieger 1986). The model assumed a binomial error distribution, calculated robust standard errors, and used a logit link function with an exchangeable correlation structure for the correlations among lobsters within a boat.

A forward stepwise procedure under the control of the investigator (i.e., not computer generated) was used to identify variables having important associations with lobster vigor at the processing plants. Initially, a model containing only the main effect for each variable showing significant association was fit. Then, all possible two-way interactions among these individually significant variables were explored. Finally, three-way interactions were considered for inclusion in the model. Models were compared using the deviance statistic, and the model with the lowest deviance was selected. The fit of the model was assessed using a Hosmer–Lemeshow goodness-of-fit test. For comparison purposes, the final model developed using GEE was subsequently fit as a multilevel model (MLwiN Version 1, University of London) with lobster as the level 1 identifier and boat as the level 2 identifier. Second-order PQL (penalized quasilikelihood) estimates were obtained using the RIGLS (restricted iterative generalized least-squares) estimation procedure.

RESULTS

Descriptive Statistics

From 64 groups of lobsters assessed during this study, 17 groups were rejected because of missing information at the processing plants. The outcome, lobster vigor upon arrival at the processing plant, was assessed on a total of 2,191 lobsters from 47 different groups or fishing boats. Because of the limited number of boats sampled in the fall and the obvious differences in environmental characteristics, only data from 38 boats in the spring season were used in developing the model. In the spring sampling, vigor was assessed on 1,181 lobsters from 38 boats. A total of 35 lobsters had decreased vigor upon arrival at processing plants in the spring season, with 12 groups having at least 1 lobster with decreased vigor.

Measure of Association

Three continuous variables (date, maximal, and minimal depths at which traps were set) and 19 of the 39 dichotomous variables were found to be significantly associated with lobster vigor (Tables 1 and 2), and retained for further analysis. The vigor of lobsters assessed directly on boats was significantly associated with vigor upon arrival at the processing plants. However, this variable was not kept as predictor for further analysis because of the small number of cases ($n = 3$) of decreased vigor observed on boats and the plant. Similarly, flatfish as bait was also dropped; although 76 lobsters were fished with flatfish as bait, it represented only 1 fisher.

Handling and Fishing Practices

The minimal and maximal depths at which traps were set showed significant unconditional associations with lobster vigor status assessed upon arrival at processing plants (Table 1). Reduced vigor lobsters tended to come from more shallow water (minimal and maximal depths of 3.5 m and 14.8 m, respectively) compared to normal vigor lobsters (minimal and maximal depths of 5.4 m and 17.6 m, respectively).

Boats with smaller crews and older captains tended to have a higher risk of producing low vigor lobsters (Table 2). Lobsters landed from boats with crews of two or fewer members were 2.4 times more at risk of having a decreased vigor at the processing plant than lobsters landed by larger crews, while the same risk was 3.0 times greater when landed by a captain with more than 20 years of experience compared to less experienced captains.

A number of weather-related variables had significant unconditional associations with lobster vigor (see Table 2). Warm weather, rain, sunlight, and rough weather (waves) all increased the risk of low vigor. After dichotomization, both the maximum (>10 °C or ≤10 °C) and minimum (>9 °C or ≤9 °C) air temperatures had significant unconditional associations with lobster vigor at processing plants. When the maximum temperature was above 10 °C, lobsters were 3.6 times more likely to experience loss of vigor at the plants than when the maximum daily temperature was 10 °C or below. A similar situation was noted with the minimal daily air temperature, with relative risk of observing decreased lobster vigor at the plant of 3.1 when the minimal air temperature was above 9 °C. When caught on rainy days, lobsters were 6.3 times more likely to have decreased vigor upon arrival at the plant compared to nonrainy days. Also significant was the exposure to sunlight, with relative risk for lobster decrease in vigor of 5.3 if landed on sunny days as compared to cloudy days. When the waves were classified as moderate to strong, lobsters were 3.3 times more likely to suffer from loss of vigor at the plants compared to when waves were calm to minimal.

Boats fishing with mackerel or flatfish baits were at higher risk of delivering lobsters with lower vigor; whereas, boats fishing with gaspereaux bait landed livelier lobsters (Table 2). When fished

TABLE 1.

Unconditional associations between fishing practices and lobster (*Homarus americanus*) characteristics that were measured on a continuous scale, and a measure of lobster vigor at the processing plant.

Variable	Description	*P*-value	Mean	
			Normal vigor[1]	Decreased vigor[1]
Date	Date of the boat sampling (# of days from January 1)	0.026	162.9	166.3
Water temp.	Surface water temperature (°C)	0.181	7.9	8.6
No. traps	Number of traps hauled	0.111	289	300
Max. depth	Maximum depth at which the traps were set (m)	0.001	17.6	14.9
Min. depth	Minimum depth at which the traps were set (m)	0.003	5.4	3.5
Weight	Individual weight as measured at the wharf (kg)	0.381	0.58	0.63
Length	Individual carapace length as measured on the boat (mm)	0.414	88.2	89.4
Protein	Total hemolymph protein, on the boat (g/L)	0.190	77.4	84.8
Hemocyte	Total hemocyte counts, on the boat ($\times 10^6$ hemocytes/ml)	0.422	198.3	221.3

[1] Normal and decreased vigor as assessed at the processing plant with the dichotomous variable "vigor."
The *P*-values were obtained by *t*-test.

with mackerel, lobsters were almost 4 times more likely to express loss of vigor compared to fishing with alternate baits, while gaspereaux had a protective effect whereby lobsters fished with gaspereaux were only one-third as likely to have reduced vigor. Lobsters fished with flatfish as bait seemed to be 5.3 times more likely to suffer loss of vigor upon arrival at the processing plant. However, flatfish bait was used by only one fishing boat included in the study and, therefore, its unconditional effect may be overestimated. For a complete description of the frequency distribution of the baits used see Lavallée et al. (2000).

Rough handling, physical contact among lobsters before measuring the carapace, the use of plastic totes as temporary storage units, and packing lobsters over at the wharves, were all practices that induced higher risk for loss of lobster vigor (Table 2). Lobsters coming from boats where physical contact before carapace measurements was possible had an 18 times greater chance of suffering loss of vigor at the processing plants than if they were landed from boats in which physical contact between lobsters was purposefully prevented. Lobsters landed from boats in which lobsters were generally tossed from the traps to the temporary holding units were almost 3 times more likely to suffer vigor loss at plants as compared to lobsters that were placed into temporary holding units. If these temporary holding units were the traditional plastic totes, then lobsters were 4 times more likely to express loss of vigor at processing plants than if other types of temporary storage units were used (Table 2). Furthermore, the practice of packing over the lobsters once at the wharf showed that lobsters that went through this process were more than 3 times more likely to have decreased vigor at the processing plants than lobsters that did not go through this process. All other handling and fishing practices did not show any significant unconditional association with the lobster vigor status at processing plants (Table 2).

Transportation Conditions

Three transportation variables individually showed statistically significant association with lobster vigor loss when assessed at arrival at processing plants: the use of closed compartment vehicles, warmer outside air temperatures, and windy conditions during transport between wharves and processing plants (Table 2). When the transport vehicle was a nonrefrigerated closed compartment truck, lobsters were almost four times more likely to have decreased vigor than if other types of vehicles (open-bed truck or pick-up truck, and vehicle equipped with a closed refrigerated transportation) were used. In the presence of moderate to strong winds during transportation, the proportion of lobsters that suffered from loss of vigor upon arrival at the processing plants was 7.3% as compared to 1.5% in the presence of calm or light winds; lobsters transported during windier days were more than five times more likely to have vigor loss at the plants. Finally, if the outside air temperature was above 18 °C, lobsters became seven times more likely to suffer vigor loss at the processing plants than if the air temperature was 18 °C or less. No other transportation conditions showed significant association with lobster vigor at the processing plants.

Regression Models

The Generalized Estimating Equation logistic regression model (GEE) identified four significant variables predicting lobster vigor at the processing plant: maximum depth at which traps were set, occurrence of rain during fishing, tossing of the lobsters from traps to temporary storage units, and the use of mackerel as bait (Table 3). While controlling for other factors, lobsters landed from boats in which mackerel bait was used instead of alternate baits were 7.1 times more likely to have decreased vigor at the processing plants; lobsters were 6.3 times more likely to have decreased vigor if landed on rainy days as compared to nonrainy days; and lobsters from boats on which they were generally tossed into the temporary holding units were 3.6 times more likely to experience vigor loss at the plant. Finally, lobsters caught in deeper waters were less likely to have loss of vigor at the processing plants than lobsters caught from shallower waters, with the risk of experiencing loss of vigor decreasing by 1.2 for every meter increase in depth, while controlling for other variables in the model.

The deviance for the multivariable model was 240.81, the Pearson dispersion coefficient for the model was 1.03, with a total of 1,148 observations, and the Chi-square value for the over-all significance of the model was 36.56 ($P < 0.001$). The standard errors were adjusted for clustering on the boat, using the GEE model. The Hosmer–Lemeshow goodness-of-fit test yielded a Chi-square value of 8.11 with a *P*-value of 0.423 (critical value = 15.507, *P*

TABLE 2.

Unconditional associations between lobster (*Homarus americanus*) handling, fishing practices, transportation conditions and lobster characteristics that were measured on a dichotomous scale, and a measure of lobster vigor at the processing plant.

Variable description	Frequency distribution if vigor[1] is decreased at the plant (%)		*P*-value	RR
Crew: size of the crew on the boat, including the captain	2 or +	28/776 (3.61%)	0.027	2.4
	3 or +	7/473 (1.48%)		
Experience: years of fishing experience of the captain	21 yrs or +	27/652 (4.14%)	0.003	3.0
	20 yrs or −	8/597 (1.34%)		
Maximum air temp.: maximum daily air temperature	11 °C or +	20/340 (5.88%)	< 0.001	3.6
	10 °C or −	15/909 (1.65%)		
Minimum air temp.: minimum daily air temperature	10 °C or +	21/412 (5.10%)	0.001	3.1
	9 °C or −	14/837 (1.67%)		
Wind: wind strength while fishing	Moderate-strong	2/201 (1.00%)	0.090	0.3
	calm-light	33/1,048 (3.15%)		
Rain: raining during fishing	Yes	21/239 (8.79%)	0.000	6.3
	No	14/1,010 (1.39%)		
Sun: sunshine during fishing	Yes	14/972 (1.44%)	< 0.001	0.2
	No	21/277 (7.58%)		
Water during: water available in the live tank, while fishing	No	26/795 (3.27%)	0.185	1.7
	Yes	9/454 (1.98%)		
Water after: water available in the live tank, after fishing	Yes	29/1,004 (2.89%)	0.709	1.2
	No	6/245 (2.45%)		
Gasperaux: fresh gaspereaux used as bait	Yes	4/400 (1.00%)	0.008	0.3
	No	31/849 (3.65%)		
Mackerel: fresh mackerel used as bait	Yes	29/700 (4.14%)	0.001	3.8
	No	6/549 (1.09%)		
Herring: fresh herring used as bait	Yes	2/127 (1.57%)	0.377	0.5
	No	33/1,122 (2.94%)		
Flatfish: fresh flatfish used as bait	Yes	6/47 (12.77%)	< 0.001	5.3
	No	29/1,202 (2.41%)		
Fiberglass: fiberglass box as live tank	Yes	5/249 (2.01%)	0.396	0.7
	No	30/1,000 (3.00%)		
X-Actic: X-Actic box as live tank	Yes	30/907 (3.31%)	0.078	2.3
	No	5/342 (1.46%)		
Tote before: plastic tote used for holding unit before grading	Yes	31/817 (3.79%)	0.012	4.0
	No	3/317 (0.95%)		
Wood box: wooden box used for holding unit before grading	Yes	0/95 (0.00%)	0.073	na
	No	34/1,039 (3.27%)		
Tote after: plastic tote used for holding unit after grading	Yes	35/1,176 (2.98%)	0.135	na
	No	0/73 (0.00%)		
Contact: physical contact among lobsters before being banded	Yes	34/895 (3.80%)	0.002	na
	No	0/239 (0.00%)		
Handling: overall lobster handling procedure on the boat	Tossed	31/859 (3.61%)	0.033	2.9
	Placed	4/322 (1.24%)		
Wave: moderate to high waves vs. small or calm sea	Moderate-strong	14/212 (6.60%)	< 0.001	3.3
	calm-small	21/1,037 (2.03%)		
Trap: combination of multiple, single, or double trap setting	Yes	35/1,176 (2.98%)	0.135	na
	No	0/73 (0.00%)		
Lid before: presence of a lid on the live tank while fishing	Yes	34/1,083 (3.14%)	0.065	5.2
	No	1/166 (0.60%)		
Time max: maximum time one lobster spent on the boat	4–6 hours	23/730 (3.15%)	0.377	1.4
	6–8 hours	12/519 (2.31%)		
Time min.: minimum time one lobster spent on the boat	>2 hours	35/1,193 (2.93%)	0.194	na
	2–4 hours	0/56 (0.00%)		
Packing: packing over of the lobsters at the wharf	Yes	3/283 (1.06%)	0.043	0.3
	No	32/966 (3.31%)		
Truck: transportation between wharf & plant in closed compartment	Yes	4/108 (3.70%)	0.018	3.7
	No	10/986 (1.01%)		
Direct: lobsters landed directly at the plant, no transport vehicle	Yes	1/239 (0.42%)	0.180	0.3
	No	13/855 (1.52%)		
Wind truck: wind strength during road transportation	Moderate-strong	21/286 (7.34%)	< 0.001	5.1
	calm-light	14/963 (1.45%)		

continued on next page

TABLE 2.

continued

Variable description		Frequency distribution if vigor[1] is decreased at the plant (%)	*P*-value	RR
Air temp truck: air temperature during road transportation	19 °C or +	29/506 (5.73%)	< 0.001	7.1
	18 °C or −	6/743 (0.81%)		
Sex: gender of the lobsters	Female	23/672 (3.42%)	0.162	1.6
	Male	12/570 (2.11%)		
Gaffkemia: gaffkemia test result, on the boat (for *Aerococcus viridans*)	Positive	2/23 (8.70%)	0.070	3.6
	Negative	12/494 (2.43%)		
Liveliness: lobsters liveliness, as assessed on the boat	Decreased	1/3 (33.33%)	0.001	12.2
	Normal	34/1,245 (2.73%)		
Wound: wound or active lesion, as assessed on the boat	Present	5/145 (3.45%)	0.617	1.3
	Absent	30/1,103 (2.72%)		
Claw: quality of the claws, as assessed on the boat	Normal	29/1,051 (2.76%)	0.832	0.9
	Abnormal	6/198 (3.03%)		
Leg: quality of the legs, as assessed on the boat	Normal	33/1,162 (2.84%)	0.768	1.2
	Abnormal	2/87 (2.30%)		
Antennae: quality of the antennae, as assessed on the boat	Normal	29/1,061 (2.73%)	0.726	0.8
	Abnormal	6/188 (3.19%)		
Body: quality of the overall body, as assessed on the boat	Normal	33/1,152 (2.86%)	0.645	1.4
	Abnormal	2/97 (2.06%)		

[1] Outcome.
The *P*-values were obtained by Chi-square tests.
RR is the relative risk associated with each variable.

≤ 0.05, $df = 8$). Therefore, it was concluded that the model was a reasonable fit for the data.

All parameter estimates from the multilevel model (results not shown) were virtually identical to those obtained from the GEE procedure. In addition, the level two (boat) variance was zero once the fixed effects in the model had been accounted for. This indicates that once the variables maximum depth at which traps were set, occurrence of rain during fishing, tossing of the lobsters from traps to temporary storage units, and the use of mackerel as bait were controlled, the probability of reduced vigor was essentially independent of the boats.

DISCUSSION

It is important to understand the difference between causation and association. A risk factor is associated with the outcome when the distribution of the outcome is significantly different between the "exposed" and "nonexposed" (Martin et al. 1987); whereas, causation must additionally include a set of guidelines to assess the likelihood of the association to be causal. Among these guidelines (but not limited to) are the following: the exposure to the risk factor must precede the outcome; the exposure to the risk factors should also be more common in the individuals expressing the outcome than in those individuals without the outcome; the removal of the risk factor should decrease the incidence of the outcome; and, the modification of the host's response should also decrease the incidence of the outcome (see Evans 1978 for a complete description of these guidelines).

Some correlation among the variables in the GEE model was present. This explains the multicollinearity problems encountered during the model building process, especially among dummy variables. Multicollinearity concerns relationships among predictor variables, but does not directly involve the outcome (Kleinbaum et al. 1988), and GEE models do not take multicollinearity problems into account. When a predictor is included in the model, adding another predictor that is correlated to the previous one contributes relatively little to the explanatory model and would seem to be nonsignificant. Care was taken during the model-building process to avoid this.

GEE models are more efficient if variables are independent (Liang and Zeger 1986). Although lobster-level predictors were clustered within fishing boats, GEE models account for factors up to two levels of clustering (Pendergast et al. 1996). However, this study did not result in any lobster-level predictors remaining in the final model.

More than 45% of all potential determinants measured showed significant crude association with the outcome, loss of vigor. Having multiple observations for every cluster; that is, multiple lobsters per fishing boat, can artificially increase the significance of many determinants (Kleinbaum et al. 1988), and a conservative approach must be taken when drawing conclusions, especially with crude associations. Although the distribution of the event of con-

TABLE 3.

Results of the generalized estimating equation regression models with 95% confidence interval to predict lobster (*Homarus americanus*) vigor at the processing plant.

Vigor	Odds Ratio	z	*P*>|z|	95% Confidence Interval	
Max depth	0.85	−3.008	0.003	−0.085	−0.018
Rain	3.63	2.538	0.011	0.294	2.286
Handling	0.28	−1.977	0.048	−2.559	−0.011
Mackerel	7.07	3.018	0.003	0.686	3.226

Max depth: maximum depth at which the traps were set (m).
Rain: if it was raining during fishing.
Handling: if the lobsters were placed (versus tossed) from the traps to the temporary storage unit.
Mackerel: if fresh mackerel was used as bait.

cern was binomial and took on only one of two values (normal or decreased vigor), the frequency of the event meant that the number of boats with decreased lobster vigor was sufficiently rare to have little statistical power if the data had been collapsed to the boat level. Because the data were clustered at the boat-level, an ordinary logistic regression would not account for this clustering (Kleinbaum et al. 1988), and would likely overestimate the significance of predictor variables. The investigators wanted to keep individual lobsters as the unit of evaluation, so both lobster-level and boat-level factors could be considered. Consequently, a GEE logistic modeling approach was chosen.

The final GEE model included four significant predictors. The significant predictors for loss of vigor at arrival at processing plants did not include any lobster-level factors, but only consisted of boat-level factors. Lobsters landed from boats using mackerel bait were more than seven times more likely to suffer from loss of vigor when arriving at the processing plant as compared to lobsters landed from boats using alternative baits, when controlling for the other variables. The biological or physiological explanation for this phenomenon is unclear. Scombroid fish, such as mackerel, contain high levels of the amino acid histidine (Nemetz and Shotts 1993). Decomposition of such fish, which is more likely to occur when carcasses are not refrigerated for extended periods, produces significant amounts of histamine by bacterial decarboxylation of the histidine (Barancin et al. 1998). Bacterial histamine contamination has been associated with adverse health effects when scombroids are consumed directly by humans (Barancin et al. 1998). If histamine is present in lobster baits, it could possibly adversely affect the health of harvested lobsters. Mackerel were not tested for histamine levels in this study, and, therefore, this is speculation without any real evidence. Further investigation of the effect of mackerel with different states of decomposition and histamine levels on lobster health would be necessary before this speculated link could be seriously considered. More recently, Castonguay et al. (1997) demonstrated the presence of small amounts of paralytic shellfish poisoning (PSP) toxins in the Atlantic mackerel. Perhaps the slight accumulation in mackerel of toxins related to paralytic shellfish poisoning may influence lobster health. It is also possible that mackerel can influence lobster health for other reasons.

Using less care in the over-all handling process of lobsters on board the boats is likely to result in loss of vigor. Tossing lobsters made them 2.9 times more likely to have loss of vigor at the processing plant than lobsters placed into the temporary storage unit, when the effect of other variables was controlled.

Groups of lobsters landed on rainy days were 6.3 times more likely to lose vigor compared to landings on days without rain. Lobsters are sensitive to fresh water exposure (Jury et al. 1994, Ennis 1995, McMahon 1995), and heavy exposure to rain will likely be detrimental for lobsters, especially for prolonged exposure times.

The odds ratio for the maximum depth at which traps were set was 0.85 for each meter. For each 3 m of additional depth at which traps were set, the lobsters became approximately 1.6 times less likely to suffer from loss of vigor upon their arrival at processing plants. Colder water temperatures found at greater depths are perhaps closer to those preferred by lobster (Crossin et al. 1998), and thus may have beneficial impacts on lobster vigor. Also, Lawton and Lavalli (1995) reported that lobsters may occasionally experience hypoxia in warm waters, especially in intertidal environments. Because of their aggressive and territorial behaviour (Aiken and Waddy 1995), perhaps some lobsters were forced to move toward less optimal habitats resulting in a precapture bias toward less healthy lobsters when caught in more shallow waters.

Lobster health predictors at the group-level (i.e., boat-level) would probably be more accessible to the industry, because they would not require individual assessments of lobsters. However, predictors at the individual (i.e., lobster-level) should yield more accurate estimates of population health, assuming a valid sample that is representative of the wild populations. None of the lobster-level parameters assessed in this study proved to be good predictors of lobster health at the processing plant, as assessed by the vigor state. Other lobster level predictors, such as physiological parameters, should be assessed to help define lobster health. The quantification of the Crustacean Hyperglycaemic Hormone (CHH) being correlated with stress levels in some decapods including the American lobster, may have potential (Chang et al. 1998, Paterson and Spanoghe 1997). Inorganic ions, such as magnesium, calcium, or potassium, metabolites and waste products such as glucose concentration and ammonia levels in the hemolymph should also be considered for further research, as proposed by Paterson and Spanoghe (1997). Although total hemolymph protein (TP) is used as a lobster health predictor by the industry, these data did not indicate that individual lobster vigor upon arrival at the processing plants was predicted by TP measurements. No association was found between low TP levels and decreased vigor at processing plants.

Perhaps the lack of correlation between the physiological parameters used in this study and the vigor state of lobsters at the processing plants was because of the lack of statistical power, with only 35 lobsters with decreased vigor in the dataset. Although the internal validity of these results is acceptable, it may not be valid for lobster health and fishing practices outside of Prince Edward Island. The lack of correlation with the outcome may also be partly explained by the utilization of a subjective outcome (lobster vigor) not sensitive enough to detect lobster stress, or loss of liveliness. Recently, Paterson (1999) suggested that analytical models may be useful in predicting certain outcomes, such as lobster weakness state or even lobster death. Epidemiological models using survival analysis methods to correlate lobster-level, boat-level, or transport-level parameters to lobster survival poststorage may be very informative, but are not appropriate for Prince Edward Island. Live lobster holding is rarely for more than 10–14 days in Prince Edward Island, and, therefore, the frequency of reduced lobster survival would probably not be sufficiently high to justify using survival models.

CONCLUSION

The main objective of this study was to establish an epidemiological model assessing the impact of different transportation, fishing, and handling practices on the health and quality of lobsters upon arrival at processing plants. Four lobster health predictors were identified, although no transportation condition factors were significantly associated. The use of different baits influenced lobster vigor with mackerel being detrimental. Further investigations of the types and quality of bait used and its influence on lobster health should be conducted. Lobster fishers should be encouraged not to toss lobsters, because gentle handling seems to enhance lobster vigor. Protection from rainy weather conditions should reduce industry losses, because lobsters directly exposed to rain experienced vigor loss. Setting lobster traps in deeper waters also seemed to be beneficial to lobster health. However, changing the water depth of traps is not readily altered by fishers because of

many other uncontrollable influences on areas available for fishing. When building such epidemiological models, it is always possible that there may be unmeasured or unmeasurable factors that are more related to the outcome (in this case, loss of vigor upon arrival at the processing plants), or that were indirectly measured with the factors included in the model-building process.

Lobsters caught in Prince Edward Island generally experience very low mortality rates preprocessing. Therefore, an examination of factors associated with survival was not feasible in Prince Edward Island. This low level of mortality is likely a result of several factors; most canner-sized lobsters (approximately 63.5–81 mm) are held in pounds for extremely short periods, and the majority of lobsters caught in Prince Edward Island waters are canner-sized lobsters. To examine handling factors on the boat and their association with survival, a similar evaluation should occur in other areas of Atlantic Canada where market-sized lobsters and weak lobsters may exist in greater proportions and where they are held in captivity for longer periods.

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FORAGING BEHAVIOR OF *CARCINUS MAENAS* (L.): COMPARISONS OF SIZE-SELECTIVE PREDATION ON FOUR SPECIES OF BIVALVE PREY

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ABSTRACT Experiments were designed to investigate size-selective predation by medium (40–55 mm carapace width) and large (55–70 mm) *Carcinus maenas* when feeding on four bivalves of contrasting shell morphologies, mussel, *Mytilus edulis*, flat oyster *Ostrea edulis*, Pacific oyster *Crassostrea gigas*, and edible cockle *Cerastoderma edule*. Medium-sized crabs preferred mussels 5–15 mm shell length (maximum shell dimension) and cockles 5–10 mm long, whereas large crabs preferred mussels 15–25 mm and cockles 10–20 mm long. Generally, no preference was shown for any particular size of either species of oyster. Comparisons amongst the preferred size ranges of prey showed that crab preference for a particular size range of prey was more strongly related to the minimum than to the maximum shell dimension, and that the minimum shell dimension was always equivalent to, or smaller than, the maximum cross section of the crabs' chelae. The size ranges of *M. edulis* and *C. edule* selected by *C. maenas* either clearly corresponded to, or were slightly smaller than, the size ranges of prey with the highest profitability (= dry weight consumed per unit of handling time). Profitability values of *M. edulis* and *C. edule*, however, showed considerable scatter; whereas those of *O. edulis* and *C. gigas* were even more variable. This variation seems to be the result of behavioral strategies by which crabs attack all encountered prey but reject those that remain unbroken after a certain number of opening attempts. Our results emphasize the mechanistic nature of size-selective feeding in *C. maenas*, and suggest that the differences in the observed patterns of size-selection were mainly determined by the contrasting morphological features of the bivalve shells, and the way these features influence the vulnerability of prey to crab predation.

KEY WORDS: Foraging behavior, *Carcinus maenas*, size-selection, bivalve

INTRODUCTION

Behavioral and mechanical aspects of predation by crabs that forage extensively on bivalve populations have been recurrent topics in research (e.g., Blundon and Kennedy 1982, Hughes and Seed 1995, Seed and Hughes 1995). Selective foraging is a major aspect within this topic, given the direct influence that the removal of certain prey types has on the abundance and distribution of the populations involved in the predatory interaction and of other species in the system that are related to them (Ebling *et al.* 1964). Many authors studying crab selective foraging behavior have related their findings to the Optimum Foraging Theorem, whereby a predator chooses its diet to maximize net energy intake per unit of handling time (Charnov 1976, Hughes 1980). Given a choice of different sizes and species of prey, a predator should select that with the highest dietary value (Pyke *et al.* 1977). However, minimisation of handling time (Hughes and Seed 1981) and the risk of claw damage associated with attacks on larger, more resistant prey items (Juanes 1992) have also been suggested as causal factors of prey selection in several crab–mollusk predator–prey relationships.

Because handling times, and, hence, prey values, have a complex variation related to the morphological characteristics of the crab chelae and the prey shell, foraging tactics can vary when crabs feed on different species of prey (Creswell and McLay 1990). Moreover, foraging strategies can also be related to differences in the patterns of prey dispersion that crabs encounter in their natural habitats (Hughes and Elner 1979). Thus, comparisons of foraging behavior when crabs are presented with different hard shelled prey can provide enlightening information regarding the basis of prey selection. Whereas numerous studies have addressed the dietary and energetic consequences of selective feeding (reviewed by Hughes 1990), few have identified the cues that make such foraging behavior possible (e.g., Kaiser *et al.* 1993).

The shore-crab, *Carcinus maenas* (L.), is abundantly distributed in the North Atlantic and is particularly common around the British Isles (Ingle 1980). Predation by shore-crabs can influence the abundance and distribution of commercially important bivalves when these are an integral part of the crab's natural diet (e.g., Dare *et al.* 1983; Sanchez-Salazar *et al.* 1987a). We investigated the foraging behavior of adult *C. maenas* when feeding on four species of bivalve mollusks with contrasting shell morphologies: the mussel, *Mytilus edulis* L.; the flat oyster, *Ostrea edulis* L.; the Pacific oyster, *Crassostrea gigas* (Thunberg); and the cockle, *Cerastoderma edule* (L). Comparisons amongst the foraging strategies adopted when feeding on these species are used to identify the stimuli involved in prey selection and to determine the basis of their size-selective feeding behavior.

MATERIALS AND METHODS

Samples of intertidal *Mytilus edulis* and *Cerastoderma edule*, covering as wide a size range as possible, were collected from naturally occurring populations at various sites around the Isle of Anglesey, North Wales. Samples of *Ostrea edulis* and *Crassostrea gigas* were obtained from CEFAS commercial oyster beds located in the Menai Strait in North Wales. Shells were cleaned of any attached fouling organisms and shell length (SL: maximum linear dimension of the shell), shell height (SH: maximum linear dimension of the axis at right angles to SL), and shell width (SW: minimum linear dimension of the shell) of each individual were measured to the nearest 0.1 mm using vernier calipers. Soft tissues were removed following brief immersion in boiling water and dried to constant weight at 60 °C. Dry tissue weight (W) was then determined to the nearest 0.01 mg on a top loading balance. Re-

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lationships between shell length (x) and width, height, and dry weight (y) were best described by the allometric equation $y = a \cdot x^b$ where a & b are constants. Linear relationships amongst these variables were obtained by least-square regressions on logarithmically transformed data. Regression lines were compared by analysis of variance using the General Linear Model with shell length as the covariate. Pairwise comparisons between the regression slopes and intercepts were then performed using Tukey's method.

Carcinus maenas 40–70 mm carapace width (CW) were collected by hand from the low shore in the Menai Strait, and maintained individually in plastic aquaria (30 × 20 cm) filled to a depth of 10 cm with running sea water. Water temperature in the aquaria varied between 12–17° C, and photoperiod was kept constant at approximately 14 h light: 10 h dark using 40 W fluorescent lights. Only undamaged male crabs in the later intermoult stage were used in the experiments in order to avoid any potential bias caused by morphological and behavioral differences associated with sex and moult stage. Following their capture, crabs were starved for 48 h before experiments in order to standardize hunger levels. Size-selection experiments were carried out by presenting medium-sized (40–55 mm CW) and large (55–70 mm CW) crabs with prey species ranging from 5–40 mm SL. Only one species of prey was offered to the crabs during any single feeding experiment. Each crab was simultaneously offered five prey items in each 5-mm size class. Prey items were scattered randomly over the floor of the aquaria and monitored twice a day. Any item consumed within each 12 h period was recorded and replaced by another of similar size in order to maintain constant prey availability. Experiments were run continuously until a consistent feeding pattern emerged (≈10 d).

The number of prey consumed within the different size classes was analyzed using a chi-square test to determine whether these deviated from random choice (Peterson and Renaud 1989). Because the number of size classes of prey offered to crabs was never less than five, in the event of chi-square tests being significant, the preferred size classes would be those consumed in >20% of the total number of prey consumed. Comparisons of the size ranges of prey preferred by both size categories of crabs were made on the basis of: (1) shell length; (2) width; and (3) relative prey size, which was obtained by dividing the median value of shell width within each of the size classes of each prey species offered by the height (maximum cross section) of the major chela. The height of the major chela in *C. maenas* was estimated using the allometric equation $MH = 0.13\ CW^{1.21}$ ($r^2 = 0.98$; $n = 61$) where MH (mm) is master chelal height and CW (mm) is carapace width (Mascaró, 1998). To compare the biomass corresponding to the total number of items of each prey species that were consumed daily by each crab, the dry flesh weight (mg) of the ingested prey within each size class was estimated from the median lengths of each size class using the appropriate allometric equations.

Handling time experiments were carried out by offering each medium-sized and large crab a prey item of known shell length, and recording: (1) breaking time (Tb), the time from the first physical contact with the prey item, through the period of manipulation to the point where the shell was finally opened and the flesh exposed; (2) eating time (Te), the period from when the prey was opened to the point where the meal was completed and the empty shell abandoned; and (3) handling time (Th; i.e., the sum of Tb and Te). If the crab was successful in the attack, another prey item was presented, and this procedure repeated until data for a wide size range of prey were obtained. If crabs were reluctant to eat, they were starved for 1–3 days until hunger levels recovered. Least-squares regressions on previously log-transformed handling times were fitted to the exponential model $y = a \cdot e^{xb}$ where a and b are constants, and handling time curves were predicted for each crab using parameters a and b. Prey profitability was estimated as dry flesh weight per unit of observed handling time (mg · sec^{-1}). Profitability curves were, therefore, obtained by dividing the estimated dry flesh weight of a prey item of known shell length by the handling time predicted by the exponential model. Analysis of variance (ANOVA) and Scheffe's method for pairwise comparisons of breaking times and profitability values between prey spe-

TABLE 1.

Equation coefficients of the allometric relationships between shell length (SL mm) and shell width (SW mm), shell height (SH mm), and dry flesh weight (W mg) in *Mytilus edulis* (*M*), *Ostrea edulis* (*O*), *Crassostrea gigas* (*C*), and *Cerastoderma edule* (*E*).

		Equation Coefficients				Turkey's Comparisons				
Relationship		a	b	r^2	n		*M*	*O*	*C*	*E*
Log SW on log SL	*M. edulis*	− 0.42	1.03	0.99	35	*M*	——	*	ns	ns
	O. edulis	− 0.23	0.70	0.82	35	*O*	*	——	*	ns
	C. gigas	− 0.39	0.96	0.97	42	*C*	ns	*	——	ns
	C. edule	− 0.35	1.15	0.99	35	*E*	*	*	*	——
Log SH on log SL	*M. edulis*	− 0.13	0.88	0.99	35	*M*	——	ns	*	ns
	O. edulis	− 0.26	1.12	0.98	35	*O*	*	——	*	*
	C. gigas	− 0.02	0.91	0.94	42	*C*	ns	*	——	ns
	C. edule	− 0.04	1.00	0.99	35	*E*	*	*	*	——
Log W on log SL	*M. edulis*	− 4.94	2.69	0.99	35	*M*	——	*	*	ns
	O. edulis	− 5.99	2.89	0.96	35	*O*	ns	——	*	*
	C. gigas	− 6.50	3.30	0.95	22	*C*	*	*	——	*
	C. edule	− 4.86	2.82	0.99	25	*E*	ns	ns	*	——

Coefficients a (intercept) and b (slope) in the linear model ($\log y = \log a + b \log x$) were obtained by least square regressions; r^2 is the coefficient of determination. Results of Tukey's pairwise comparison tests performed on the regression coefficients (slopes: normal type; intercepts: bold type) of each allometric relationship are also presented: $^*P < 0.05$, ns = not significantly different.

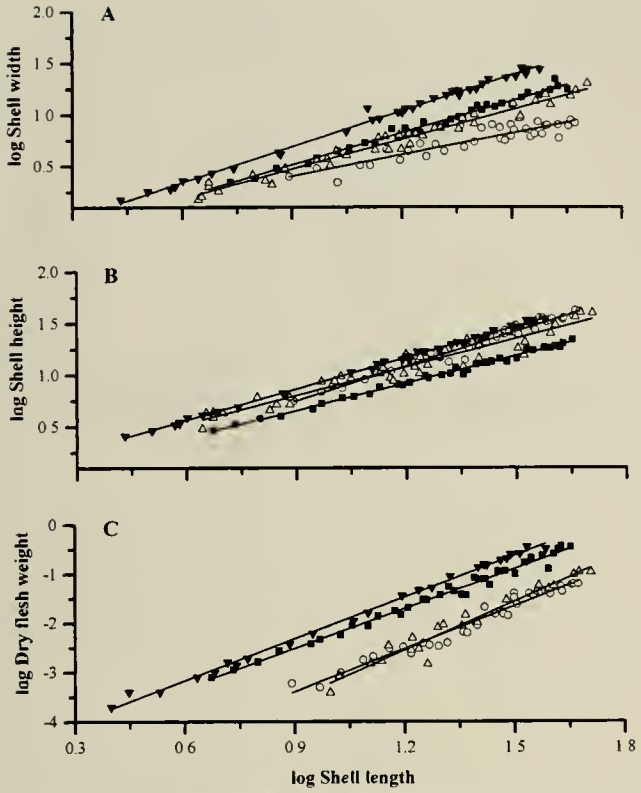

Figure 1. Relationships between (A) shell width (mm), (B) shell height (mm), (C) dry flesh weight (mg) and shell length (mm) for a wide size range of *Mytilus edulis* (solid squares), *Ostrea edulis* (open circles), *Crassostrea gigas* (open triangles), and *Cerastoderma edule* (solid triangles). Solid lines represent predicted values based on the parameters of the allometric equations presented in Table 1.

cies were performed on the basis of the size ranges of prey preferred by crabs during the size-selection experiments. In those cases where crabs exhibited no apparent size preference, the size range used was comparable to that for the preferred size range of mussels. Breaking times and profitability values were log-transformed before analysis of variance was applied to the data.

RESULTS

Analysis of variance of the allometric relationships between shell length and shell width, height and dry flesh weight revealed significant differences amongst the four bivalve species (Table 1). Analysis of variance showed significant differences amongst the slopes ($F = 34.12$; $P < 0.001$) and intercepts ($F = 2.87$; $P < 0.05$) for the regressions between shell width and length of the four species examined (Table 1). With increase in shell length, *C. edule* increased in shell width more rapidly than *M. edulis* and *C. gigas*, which in turn increased in shell width more rapidly than *O. edulis*. Analysis of variance on the regression lines of shell height and length showed significant differences amongst the slopes ($F = 11.5$; $P < 0.001$) and intercepts ($F = 6.88$; $P < 0.001$). With increase in shell length, *O. edulis* increased in shell height more rapidly than *C. edule*, which, in turn, increased more rapidly than *M. edulis* and *C. gigas*. Thus, among small individuals shell width and height of the four bivalves were broadly similar. However, among larger prey, *C. edule* had a significantly wider shell than both *M. edulis* and *C. gigas* of comparable shell length; whereas, *O. edulis* had the narrowest shell of the four species (Fig. 1A). Large *O. edulis* and *C. edule*, however, had significantly higher shells than both *M. edulis* and *C. gigas* of comparable length (Fig. 1B).

Results of the analysis of variance on the regression lines of weight and shell length showed significant differences among the slopes ($F = 6.82$; $P < 0.001$) and intercepts ($F = 46.92$; $P < 0.001$). Pairwise comparisons between regression parameters revealed that the regression lines for *M. edulis* and *C. edule* are not significantly different from each other (Table 1), and that both have the same slope but a higher elevation than the regression line for *O. edulis*. These results suggest that both *M. edulis* and *C. edule* had significantly more biomass than any *O. edulis* of comparable shell length (Fig. 1C). The regression line for *C. gigas* intersects that for *O. edulis*, suggesting that among smaller prey, Pacific oysters had less biomass than mussels, cockles, and flat oysters. However, with increase in size, the biomass of *C. gigas* increased more rapidly than in the other species (slope: 3.30 ± 0.34) so that among prey > 20 mm long, *C. gigas* had more flesh than *O. edulis* of comparable shell length.

Both the degree of crab selectivity (i.e., pattern of size selection) and the maximum length of prey consumed by *C. maenas* varied from one prey species to another (Fig. 2). Although the percentage of mussels consumed by all crabs decreased slowly among mussels of increasing shell length, the percentage of cockles consumed declined steeply among the larger size classes of prey. The maximum shell length of *M. edulis* opened by medium-

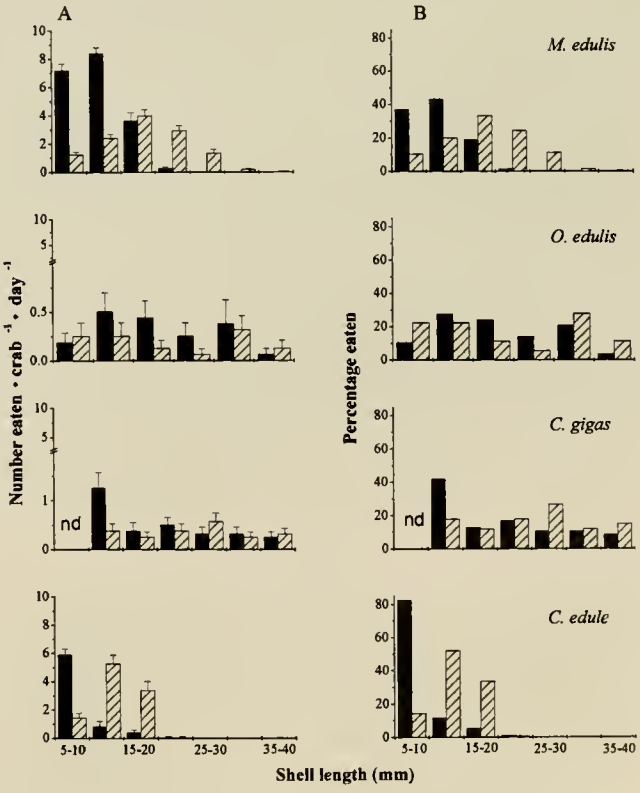

Figure 2. (A) Number (± se) and (B) percentage of *Mytilus edulis*, *Ostrea edulis*, *Crassostrea gigas*, and *Cerastoderma edule* that were consumed daily by each *Carcinus maenas* 40–55 mm CW (black columns) and 55–70 mm CW (hatched columns) during a period of 8–10 days. Note that *C. gigas* 5–10 mm in shell length were not available during these experiments (nd = no data).

TABLE 2.

Results of chi-square (χ^2) tests on the total number of prey consumed by medium (40–55 mm CW) and large (55–70 mm CW) *Carcinus maenas* during size-selection experiments.

	Medium				Large			
		Preferred Size Ranges				Preferred Size Ranges		
Species	χ^2	SL	SW	RPS	χ^2	SL	SW	RPS
M. edulis	699.5*	5–15	2.0–6.3	0.15–0.46	235.9*	15–25	6.3–10.6	0.33–0.56
O. edulis	7.2 ns	——	——	——	4.0 ns	——	——	——
C. gigas	22.8*	10–15	3.7–5.4	0.27–0.40	3.1 ns	——	——	——
C. edule	282.6*	5–10	2.9–6.4	0.21–0.47	165.1*	10–20	6.4–14.1	0.34–0.75

RPS was calculated as the median value of shell width in each size class of prey divided by the height of the master chela in each size category of crab. The preferred (i.e., consumed in >20%) size ranges of each prey species are expressed in terms of shell length (SL mm), shell width (SW mm) and relative prey size (RPS) when chi-square tests proved statistical significance; *significant at $P < 0.001$, ns = no significant departure from a random choice.

sized and large crabs was 20–25 and 35–40 mm, respectively; whereas, the maximum shell length of *C. edule* opened by both size categories of crabs was 20–25 mm. By contrast, crabs included *O. edulis* and *C. gigas* of up to 35–40 mm in their diet and were less size selective than when feeding on mussels and cockles, resulting in relatively more uniform distributions in both oyster species.

Chi-square analysis on the number of prey consumed by each size category of crab revealed that medium-sized crabs (40–55 mm CW) significantly preferred mussels of the two smallest size classes (5–15 mm SL), and cockles of the smallest size class offered (5–10 mm SL; Table 2). Larger crabs (55–70 mm CW) showed a significant preference for slightly larger mussels (15–25 mm SL) and cockles (10–20 mm SL). Neither medium nor large crabs included cockles >25 mm long in their diets. When feeding on oysters, crabs generally showed no preference for any particular size class of either species, the only exception being medium-sized *C. maenas* that consumed *C. gigas* 10–15 mm long in significantly higher numbers, but included Pacific oysters of all size classes offered. Differences between the preferred size ranges of prey were larger when expressed in terms of shell length than in terms of shell width. For example, medium *C. maenas* preferred mussels 5–15 mm and cockles 5–10 mm long, but these measured 2.0–6.3 mm and 2.9–6.4 mm in shell width, respectively. In addition, crabs always preferred prey with a relative prey size <1, suggesting that preferred prey was always smaller than the height of the largest chela.

The total number of each prey species consumed daily by each size category of crab and the corresponding biomass (mg) varied from one prey species to another (Table 3). *Carcinus maenas* consumed more mussel flesh than any of the other bivalves offered, and differences in consumed biomass were greatest between *M. edulis* and *O. edulis*. There was a general trend toward higher biomass consumption among crabs 55–70 mm CW, but these larger crabs generally consumed fewer prey items than crabs 40–55 mm CW, presumably because large crabs fed on larger prey and these would have proportionately greater biomass.

Handling times and profitability curves for each size category of crab feeding on each prey species and the estimated parameters of the exponential relationships varied among the four bivalve prey (Table 4, Fig. 3. The strong reluctance of *C. maenas* to feed on large *C. edule* did not allow for handling curves to be predicted for cockles >20 mm long. However, handling times for small cockles were overall much lower than for any of the other prey species. From the handling time data it is clear that large crabs required less time to handle prey of any particular shell length than did medium-sized crabs, and, consequently, had access to larger prey items of any of these prey species. Results showed a considerable scatter within the handling time data for each prey species, and this became more extreme when profitability values were plotted, particularly among crabs feeding on *O. edulis* and *C. gigas*. Nonetheless, profitability plots suggest that larger crabs were consistently capable of obtaining higher profitability than medium-sized crabs, regardless of the size and species of prey offered. There also seemed to be an optimum size of prey, below and above which

TABLE 3.

Ingested dry biomass (mg · crab^{-1} · day^{-1}) corresponding to the total number (No.) of *Mytilus edulis*, *Ostrea edulis*, *Crassostrea gigas*, and *Cerastoderma edule* of all size classes consumed by medium (40–55 mm CW) and large (55–70 mm CW) *Carcinus maenas*.

	Medium		Large	
Species	Biomass	No.	Biomass	No.
M. edulis	211.2	19.4	417.6	12.0
O. edulis	11.8	1.8	9.2	1.1
C. gigas	34.5	3.0	37.2	2.1
C. edule	60.4	7.1	252.5	10.1

TABLE 4.

Equation parameters for the exponential relationships between handling time (Th sec) and shell length (SL mm) for medium (40–55 mm CW) and large (55–70 mm CW) *Carcinus maenas* feeding on a size range of *Mytilus edulis*, *Ostrea edulis*, *Crassostrea gigas*, and *Cerastoderma edule*.

	Medium				Large			
Species	a	b	r^2	n	a	b	r^2	n
M. edulis	2.93	0.24	0.87	50	2.41	0.21	0.80	33
O. edulis	1.94	0.21	0.74	26	3.28	0.14	0.56	15
C. gigas	2.99	0.18	0.81	26	3.18	0.14	0.90	21
C. edule	3.27	0.19	0.69	23	2.21	0.24	0.68	24

a and b were estimated as: ln Th = ln a + b SL; r^2 is the coefficient of determination.

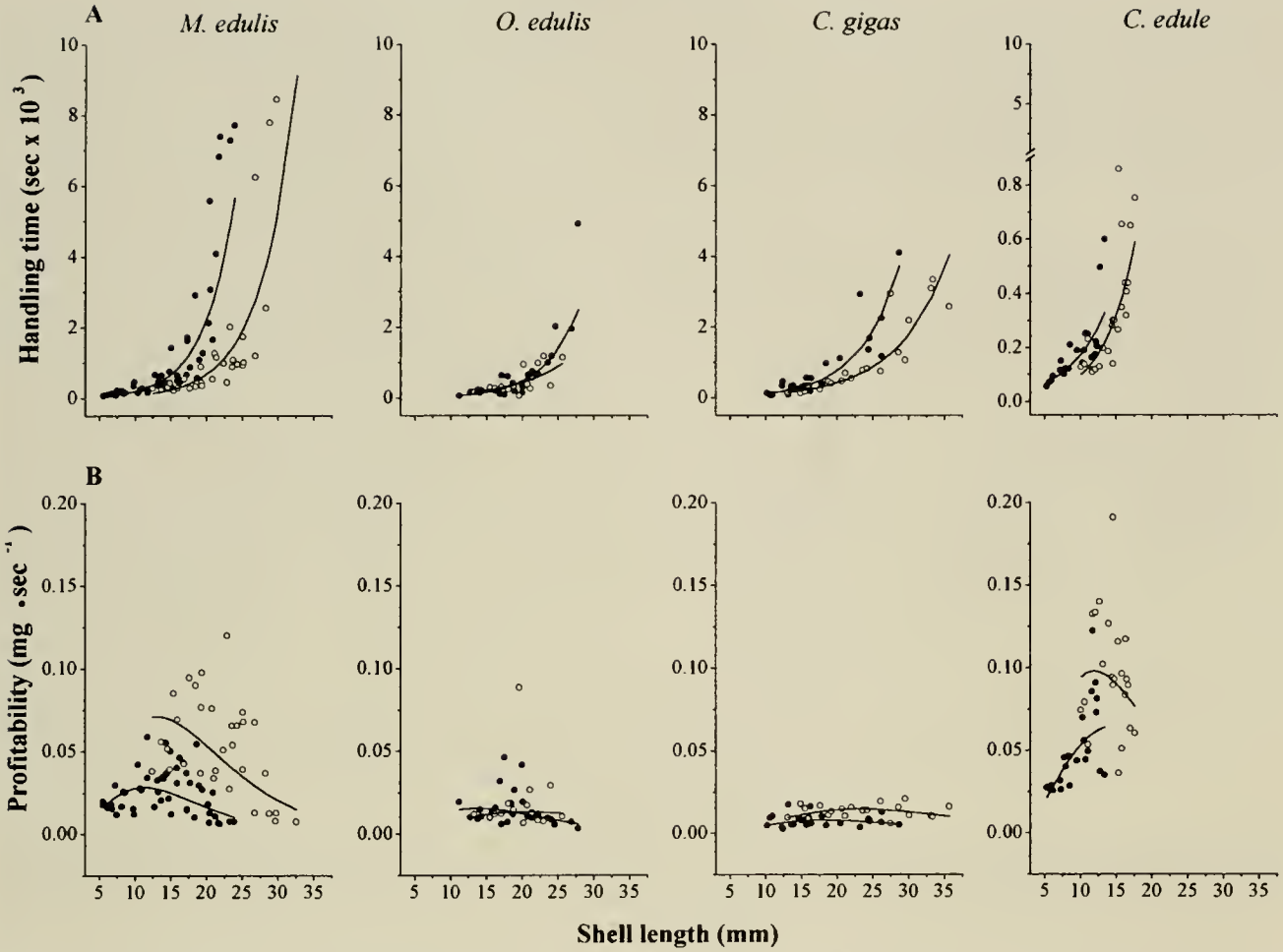

Figure 3. (A) Handling time and (B) profitability for a size range of *Mytilus edulis*, *Ostrea edulis*, *Crassostrea gigas*, and *Cerastoderma edule* consumed by *Carcinus maenas* 40–55 mm CW (solid symbols) and 55–70 mm CW (open symbols). Solid lines represent predicted values based on the parameters of the exponential equations presented in Table 4, and on the dry weight-shell length relationships presented in Table 1. Note scale change for *C. edule* handling time.

profitability decreased, and this optimal size seemed to increase as a function of crab size. In both oyster species, however, the size range at which profitability was maximized was wide, and peaks were relatively broad. Both medium and large *C. maenas* obtained slightly higher profitability when feeding on cockles than on mussels of similar shell length, and crabs would, therefore, need to feed on slightly larger mussels than cockles to obtain similar profitability.

The ranking order of prey profitability paralleled the order in which prey were consumed by both size categories of crabs during feeding experiments (Fig. 4). Analysis of variance showed that profitability of the preferred size range of *M. edulis* was significantly greater than that of *O. edulis* and *C. gigas* (Table 5). The preferred size ranges of mussels and cockles, however, provided similar profitability for crabs in both size categories. No significant differences were detected between profitability of the preferred size classes of *O. edulis* and *C. gigas* for either medium or large crabs. Analysis of variance of breaking time of the preferred size range of each prey species revealed that medium-sized crabs took significantly less time to break open cockles than mussels of the preferred size range (Table 5). Among large crabs, however, significant differences were only found between prey with extreme values (*C. gigas* > *C. edule*). No significant differences were ever found between the breaking times of mussels and oysters or between the two oyster species. Because crabs generally took similar times to open all four bivalves, these results suggest that differences in profitability between the selected size ranges of prey were mainly because of differences in their biomass.

DISCUSSION

Optimal Foraging Theory assumes that predators are able to rank prey in the order of their dietary value, and predicts that prey should be selected accordingly (Charnov 1976, Pyke *et al.* 1977, Hughes 1980). When, in the present study, *Carcinus maenas* were fed *Mytilus edulis* and *Cerastoderma edule*, there was an optimum size range of prey above and below which profitability (= dry flesh weight ingested per unit of handling time) decreased (Fig. 3). Moreover, the size ranges of *M. edulis* and *C. edule* actually selected by these crabs either closely corresponded to, or were slightly smaller than, the size ranges of prey with the highest profitability (Table 2). Results similar to these have previously been demonstrated for *C. maenas* when feeding on mussels (Elner and Hughes 1978) and cockles (Sanchez-Salazar *et al.* 1987b).

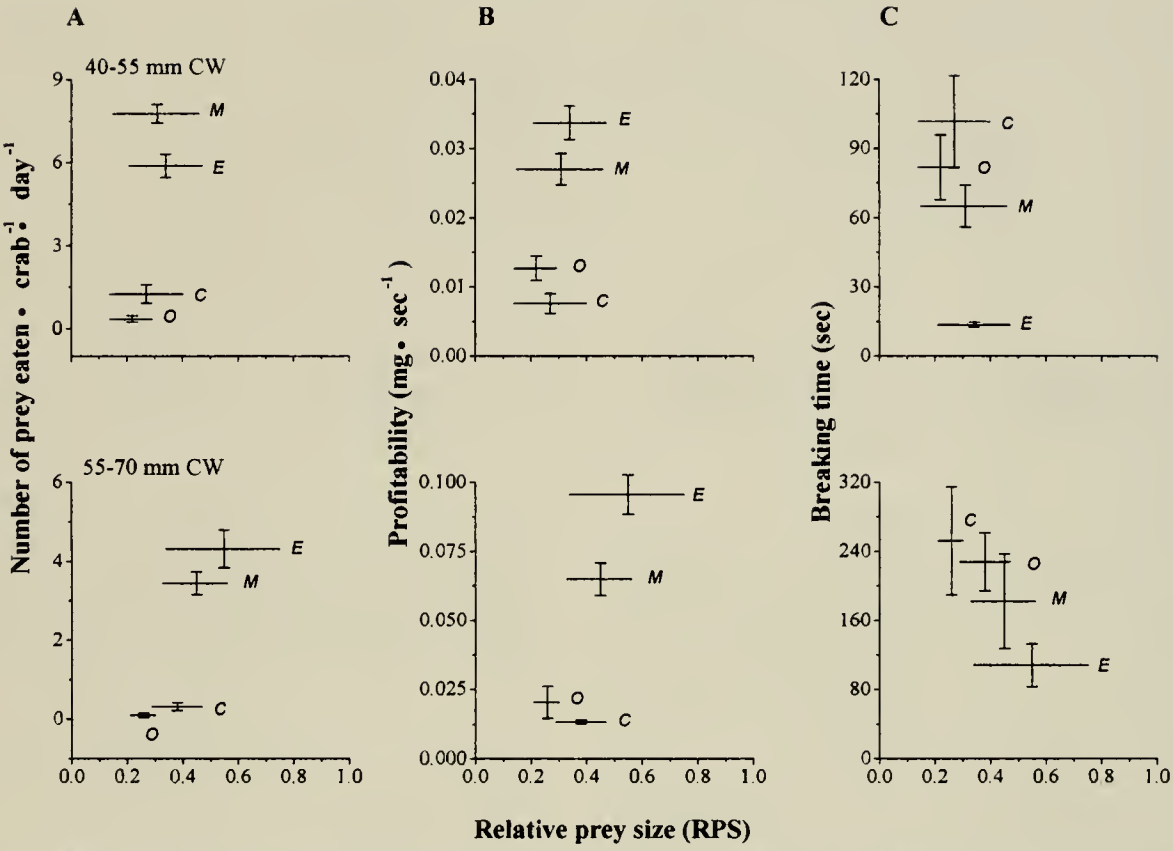

Figure 4. (A) Mean number (± se), (B) profitability (± se) and (C) breaking time (± se) of *Mytilus edulis* (*M*), *Ostrea edulis* (*O*), *Crassostrea gigas* (*C*), and *Cerastoderma edule* (*E*) of the size range that were preferred by *Carcinus maenas* 40–55 mm CW (upper panels) and 55–70 mm CW (lower panels). For comparative purposes prey size is expressed as relative prey size (RPS) = shell width/chelal height.

TABLE 5.

Results of analysis of variance and selected pairwise comparisons using Scheffe's method on profitability and breaking time data of *Mytilus edulis* (M), *Ostrea edulis* (O), *Crassostrea gigas* (C) and *Cerastoderma edule* (E) consumed by medium (40–55 mm CW) and large (55–70 mm CW) *Carcinus maenas*.

Size category	*F*	Pairwise Comp.	Diff. Mean	SE	LCI	HCI	*P*	Result
Profitability								
40–55 mm	30.98	M-O	0.31	0.089	0.05	0.56	*	M > O
	***	M-C	0.58	0.070	0.37	0.78	*	M > C
		O-C	0.27	0.102	−0.03	0.56	ns	O = C
		M-E	−0.12	0.065	−0.31	0.07	ns	M = E
55–70 mm	60.00	M-O	0.60	0.072	0.39	0.80	*	M > O
	***	M-C	0.66	0.078	0.44	0.89	*	M > C
		O-C	0.07	0.083	−0.17	0.305	ns	O = C
		M-E	−0.17	0.062	−0.35	0.004	ns	M = E
Breaking time								
40–55 mm	16.92	M-O	−0.17	0.138	−0.57	0.23	ns	M = O
	***	M-C	−0.23	0.109	−0.54	0.09	ns	M = C
		O-C	−0.05	0.158	−0.51	0.41	ns	O = C
		M-E	0.58	0.101	0.29	0.87	*	M > E
55–70 mm	4.30	M-O	−0.19	0.136	−0.58	0.20	ns	M = O
	**	M-C	−0.24	0.148	−0.66	0.19	ns	M = C
		O-C	−0.05	0.157	−0.50	0.40	ns	O = C
		M-E	0.18	0.118	−0.16	0.52	ns	M = E

Diff. Mean = difference between means; SE = standard error of the mean; LCI = low limit of confidence interval; HCI = high limit of confidence interval; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$; ns = no significant differences between pairs of variables.

Elner and Hughes (1978) suggested that the feeding strategy exhibited by *C. maenas* maximized net energy intake; whereas, Sanchez–Salazar *et al.* (1987b) suggested that preference for smaller size classes of cockles probably reflected shorter absolute breaking times.

Profitability values in the present study, however, showed considerable variation, particularly from the preferred to the largest size ranges of prey consumed (Fig. 3). Examination of the data showed that small differences in shell length could result in large variations in handling time, suggesting that differences in the thickness and fracture resistance between individual prey items of similar linear length probably accounted for much of the observed variation in handling time and profitability. Moreover, the patterns of variation seem to be the result of behavioral strategies that crabs present when feeding on prey that vary little in size, yet vary markedly in vulnerability. When crabs opened smaller prey within the size range offered, breaking time was approximately constant, indicating that crushing techniques used by crabs were equally effective on all small size classes of these bivalves. This resulted in short handling time but low profitability values, although eating time, and hence handling time, constantly increased with prey size as a result of small increments in flesh weight. When crabs attempted to open a prey item of an intermediate size, the shell would sometimes yield in a short period of time, making that particular prey item highly profitable because of its relatively larger biomass. However, when the shell did not yield in the first few crushing attempts, crabs took a considerably longer time to access the flesh, resulting in reduced profitability. Microfractures in the shell substructure and possible leakage of body fluids as the shell started to break probably constituted a reinforcing cue for crabs, causing them to persist with that prey item, thereby increasing substantially handling time. Thus, prey of an intermediate but similar shell length had extremely variable profitability, although among these prey, profitability achieved its maximum value.

Because flesh weight increases approximately as the cube of shell length (Table 1); whereas, handling time increases exponentially with respect to shell length (Table 4), the time taken by crabs to handle prey successfully ultimately increased more rapidly than the biomass obtained. The lowest profitability values, therefore, generally corresponded to prey items of a larger shell length, which, although offering the greatest biomass, could only be opened by crabs using more complex and time-consuming handling techniques.

Variations in breaking time of a narrow size range of prey could be attributable to differences in hunger levels (Hughes and Elner 1979), or to learning mechanisms developed by crabs in order to manipulate prey of a particular shape (Cunningham and Hughes 1984). Age-specific differences in shell strength and morphology of individual prey items can also influence size-related preferences (Boulding 1984). Our results indicate that the lack of precision in predicting prey value could be attributable to one or more of these factors rather than to experimental designs that fail to measure handling time accurately. Furthermore, our results suggest that size selection may be the result of a mechanical process in which all encountered prey items are attacked but rejected if they remain unbroken after a certain number of opening attempts. If this latter view is correct, the decreasing order of crab preference for different size classes of prey should reflect the decreasing order of their vulnerability to crab attack. A pattern of size selection that decreases monotonically with increase in prey size corresponds to the behavioral strategy described above, and has been reported for several brachyuran crabs feeding on a variety of hard-shelled molluscan prey (e.g., Pearson *et al.* 1981, Davidson 1986, Juanes and Hartwick 1990). Moreover, authors have suggested that selection of small size classes of hard-shelled prey can minimize handling time (Hughes and Seed 1981, Seed 1990) as well as the risk of claw damage (Juanes and Hartwick 1990), thereby increasing survival of foraging crabs that may themselves be vulnerable to predation.

In the present study, however, large *C. maenas* selected intermediate size classes of *M. edulis* (Fig. 2). Earlier studies have reported that larger prey items may be less preferred, because of their robustness to crab attack; whereas, the smaller size classes of prey are less preferred simply because they are encountered less frequently, misidentified among shell debris, or frequently dropped (e.g., Elner and Hughes 1978, Rheinallt and Hughes 1985). This latter would be most likely for crabs with large, less dextrous chelae that do not allow for the efficient manipulation of small prey items. Although our study did not include experiments that presented crabs with altered proportions of different size classes of prey, observations during handling time experiments showed that large crabs often had difficulty encountering, identifying, and grasping small prey items.

Variability in handling time and profitability was even more pronounced among *C. maenas* feeding on oysters, and the size ranges of *Ostrea edulis* and *Crassostrea gigas* at which profitability attained its highest values were not clearly delimited (Fig. 3). The precise orientation of the shell within the chelae when force is first applied further influenced oyster handling time, presumably as a result of their irregular shape and resistance at different points of the shell. Furthermore, large variations in oyster profitability probably explain the lack of size-related preferences by crabs when feeding on these particular bivalves. If crabs attempted to open oysters as they encountered them, rejecting those that did not yield to the first few crushing attempts, then the oysters consumed would be those that took the least amount of time to open. Such a feeding strategy would eventually result in the more uniform patterns of size selection we observed for both *O. edulis* and *C. gigas*. Our results, therefore, emphasize the mechanistic nature of size-selective feeding in *C. maenas*. Because the diets of crabs feeding on all four prey species corresponded approximately to those predicted by optimal foraging theory, a behavioral strategy by which crabs attack all encountered prey but reject those that remain unbroken after a certain number of crushing attempts probably maximizes feeding efficiency.

Despite the variability in handling time, profitability curves varied among the species of bivalve prey used; the patterns of size selection similarly varied from one prey species to another. These results strongly suggest that differences in size-selective predation among these bivalve prey are related to the contrasting morphological features of their shells and the way these features influence the vulnerability of such prey to predation by shore crabs. Not only do these four bivalves have contrasting shell shapes, but, as they increase in size, their flesh content increases at different relative rates (Table 1). Differences in the total biomass consumed by crabs when offered each of these four prey species individually partly reflects the variations in their flesh content (Table 3). However, differences in the total number of prey items consumed suggest that consumption rates among prey species also reflect crab feeding preferences.

Sanchez-Salazar *et al.* (1987b) attributed differences in the size selection of *M. edulis* and *C. edule* by *C. maenas* to variations in

shell morphology and strength per unit length. These authors showed that the shell dimensions of cockles that could be opened by crabs of a given chelal strength were less, but the energy obtained was greater than when feeding on mussels. Accordingly, they suggested that crabs could obtain better yields by consuming cockles than mussels of a similar linear size. In the present study, profitability values for *C. maenas* feeding on cockles were over-all higher than when feeding on mussels (Fig. 3). However, the size classes of both prey species that were selected by crabs yielded similar biomass per unit time (Table 5, Fig. 4). Crabs of both size categories preferred cockles of a shell length that was slightly smaller than for the preferred mussels, but the preferred size ranges of both species were of similar shell width (Table 2). In addition, the percentage of prey consumed by all crabs decreased more steeply for cockles than for mussels among the larger size classes of prey (Fig. 2), suggesting that the ability of crabs to crush prey decreased more abruptly for cockles than for mussels as these increased in size. As the more globular-shaped cockles increase in length, shell width increases more rapidly than in the more elongate mussels; consequently, cockles have a significantly wider shell than mussels of similar shell length (Fig. 1). Because dome-shaped shells are intrinsically stronger than flatter shells (Wainwright 1969), the presence of a higher dome in the more convex cockle shell probably increased force applications required by crabs to open this infaunal bivalve. Such shell features as large size, increased thickness, greater inflation, and the absence of gape reduced the vulnerability of clams to predation by *Cancer productus* (Boulding 1984), and have been shown to influence size-related preferences of crabs (Blundon and Kennedy 1982, Seed 1993, Walne and Dean 1972) and other decapods (Griffiths and Seiderer 1980) feeding on bivalve prey.

The importance of shell shape and volume also became evident in our experiments with *O. edulis*. As prey increased in length, *O. edulis* shells became significantly higher, but much narrower than *M. edulis, C. edule,* and *C. gigas* (Fig. 1). The total shell height of *O. edulis* constituted a shape-related restriction that made crabs unable to accommodate the shell within the widest aperture of the chelae, while its smaller width prevented crabs from firmly grasping the shell in a horizontal position and applying an effective crushing force. The shape-related restriction imposed by the shell dimensions of *O. edulis* resulted in similarly long handling times over all the size ranges of oysters offered, thus explaining the lack of size selection by *C. maenas* (Fig. 2).

The patterns of oyster size-selection in our experiments accord with those previously reported for *C. maenas* when feeding on *C. gigas* and *Tiostrea (= Ostrea) lutaria* (Richardson *et al.* 1993); the latter, as with *O. edulis,* has a very flat shell. Similarly, *C. maenas* has been reported to open *C. gigas* of up to 50–60 mm SL (Dare *et al.* 1983). Although Richardson *et al.* (1993) suggested that the reluctance of *C. maenas* to feed on *T. lutaria* was perhaps related to characteristics in the shape of its shell, Dare *et al.* (1983) explained their own results in terms of the presence of thin "window" areas in the valves of *C. gigas,* which made these oysters particularly vulnerable. Our results, therefore, emphasize the importance of shell shape in determining the vulnerability of different prey species and suggest that crab preference for a particular size range of prey is more strongly related to the minimum, than to the maximum, linear dimension of the shell. As bivalves grow, their increased minimum shell dimension makes them too large to be accommodated completely within the chela, decreasing the amount of force that can be applied (Boulding 1984). Moreover, results expressed in terms of relative prey size showed that the size of prey preferred by *C. maenas* were all within the range of 0.15–0.75. The initial position of the prey within the chela during the first application of force was always with the widest, most voluminous part of the shell in the region where the dactylus and propus close to form a distinct gape. If crabs selected prey that yielded in the shortest time relative to their crushing efforts, then it is likely that they would consistently select those prey with a shell width that is equivalent to, or smaller than, the maximum height of their major chela. This is not surprising, because size-selective feeding is strongly related to chelal height (Seed and Hughes 1997) and relative prey size reflects the importance of the shape, volume, and position of prey when handled by crabs in diverse attack strategies. Thus, when crab foraging behavior is being examined, prey size based solely on shell length is not an appropriate indicator of the shell characteristics associated with crab preference; the geometry and crushing resistance of prey shells should also be taken into account.

Interspecific differences in shell shape and thickness between *Littorina rudis* and *L. nigrolineata* determined prey vulnerability to predation by *C. maenas* (Elner and Raffaelli 1980), and are, therefore, expected to influence crab preference among other prey species. Whether *C. maenas* exhibits a species-selective feeding behavior regarding these bivalve prey and whether this behavior involves an active choice will be addressed in a subsequent publication. However, in this study, crabs consumed the four prey species at different rates (Fig. 2), and the order in which prey species ranked according to consumption rates clearly paralleled the rank order of prey profitability (Fig. 4). Because differences in profitability between the selected size ranges of prey were attributable mainly to differences in their biomass, these results provide an indication that prey value could influence prey species-selection by *C. maenas*.

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FORAGING BEHAVIOR OF *CARCINUS MAENAS* (L.): SPECIES-SELECTIVE PREDATION AMONG FOUR BIVALVE PREY

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ABSTRACT Species-selective predation by medium (40–50 mm carapace width) and large (55–70 mm) *Carcinus maenas* was investigated by presenting individual crabs with paired combinations of mussels *Mytilus edulis*, flat oysters *Ostrea edulis*, Pacific oysters *Crassostrea gigas*, and cockles *Cerastoderma edule* in various proportions. When offered mussels and either oyster species simultaneously, both size categories of crabs consistently selected mussels, and food choice was independent of prey relative abundance. By contrast, *C. maenas* selected mussels and cockles as expected by the frequency in which each size category of crab encountered the preferred size ranges of prey. Crab preference clearly paralleled the rank order of prey profitability, which, in turn, was mainly determined by prey biomass, suggesting that active selection takes place at some point of the predation cycle. Decisions by crabs on whether to attack oysters and mussels initially were not influenced by the flavor or odor of their flesh. Both mussel and oyster filtrates had a similar reinforcing effect on crab perseverance to open prey. However, species-related preferences exhibited by crabs feeding at or near the optimal size suggest that foraging decisions are partly based on evaluations of over-all prey shape and volume, and that the minimum dimension of the shell constitutes an important feature that crabs recognize and associate with prey value.

KEY WORDS: Foraging behavior, *Carcinus maenas*, species-selection, bivalve

INTRODUCTION

Patterns of prey selection are the result of a sequence of specific behavioral components that a predator performs during a predation event, including the location, attack, capture or attack success, and ingestion, of prey (Hughes 1980). Feeding preferences can be partially attributable to passive or mechanistic consequences of physical properties in a predator–prey system that determine encounter rate and prey vulnerability (Rodrigues *et al.* 1987; Sponangle and Lawton 1990). However, decisions by predators on whether to attack an encountered prey item or to continue searching, or to reject or accept a prey item that is being manipulated, are still the result of an active choice (Barbeau and Scheibling 1994; Sih and Moore, 1990). To comply with Optimal Foraging Theory, these decisions must be based in part on the ability to recognize prey characteristics that correlate with profitability, defined as the potential energy yield per unit of handling time. Mechanisms of prey recognition are relatively well understood among visual hunters such as fish (e.g., Ibrahim and Huntingford 1989). Crabs, on the other hand, have been the subject of comparatively few works relating these mechanisms to optimal foraging behavior (e.g., Kaiser *et al.* 1993).

Although size-selective predation in the common shore-crab, *Carcinus maenas* (L), has been extensively documented (e.g., Elner and Hughes 1978, Elner and Raffaelli 1980, Jubb *et al.* 1983), little information is available for selective predation amongst different species of prey. This paper examines the prey species-related preferences exhibited by *C. maenas* when feeding on a variety of combinations and proportions of the mussel, *Mytilus edulis* L., the flat oyster, *Ostrea edulis* L., the Pacific oyster, *Crassostrea gigas* (Thunberg), and the cockle, *Cerastoderma edule* (L). A series of experiments testing the importance of shell shape and flesh odor/flavor in prey species selection was designed to investigate which of these characteristics determined crab decisions throughout a foraging bout.

MATERIALS AND METHODS

Species-selection experiments were performed by presenting medium (40–55 mm carapace width: CW) and large (55–70 mm CW) *C. maenas* with paired combinations of a wide size range of prey species (Table 1). Five prey items in each 5 mm-size class were scattered randomly over the floor of the aquaria and monitored twice a day. Any item consumed within each 12-h feeding period was recorded and replaced by another of similar size to maintain constant prey availability. Experiments were run continuously until a consistent feeding pattern emerged (≈10 d). Crabs and prey were collected and maintained as described in Mascaró and Seed (2000).

To establish whether crab preferences resulted from a passive response to the rate in which the prey species were presented, medium and large crabs were individually offered equal and unequal numbers of the preferred size ranges of prey in the combinations *M. edulis–O. edulis* and *M. edulis–C. edule*. The size classes of preferred prey were established on the basis of those most frequently consumed in single prey species experiments (Mascaró and Seed, 2000; Table 1) The proportions of presented prey were altered prey so that the prey species that had been preferentially selected in the previous experiments was now at the lower relative abundance of 1:2 and 1:4 with respect to the less preferred species. Each time a prey item was consumed by a crab it was immediately replaced by another of similar size, and the precise order in which prey items were taken was recorded. Once a prey item was encountered and recognized as potential food, a crab could either reject (i.e., touch, manipulate and finally abandon) or accept (i.e., successfully open and consume) the prey. Because satiation, among other factors, can strongly influence the behavioral sequence adopted by an individual crab, each trial was run for 1 h, the average period of time taken for crabs to become

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TABLE 1.

Size ranges (mm in maximum shell dimension) of *Mytilus edulis* (*M*), *Ostrea edulis* (*O*), *Crassostrea gigas* (*C*), and *Cerastoderma edule* (*E*), that were offered to two size categories (mm of carapace width, CW) of *Carcinus maenas* in experiments with a wide size range of prey, and with equal and altered proportions of prey species.

Experiment	CW	*M–O*	*M–C*	*O–C*	*M–E*
Wide size	40–55	5–30	10–40	10–40	10–40
range of prey	55–70	5–30	10–40	10–40	10–40
		M	*O*	*C*	*E*
Equal and altered	40–55	10–15	10–15	10–15	5–10
proportions	55–70	15–20	15–20	15–20	10–15

Size classes were established on the basis of those most frequently selected by crabs in experiments where prey were presented individually (Mascaró & Seed 1999).

satiated during preliminary observations. Experiments were repeated on a daily basis until a consistent pattern emerged (≈5 d). The total number of times that crabs encountered each prey species were then tested for goodness-of-fit to the expected values (assuming a probability of encounter: 1:1, 1:2, and 1:4). Differences in the total number of prey of each species that were successfully opened by each individual crab were tested using the same procedure.

Two experiments were designed to examine whether preference for a certain prey species was influenced by the odor/flavor of the flesh. First, five individually maintained *C. maenas* (50–60 mm CW) were presented with 10 agar cylinders (9 × 10 mm), 5 mussel-flavored and 5 oyster-flavored. Gels were made using a filtrate of 10 g of either mussel or oyster flesh homogenized in 100 mL of seawater; this was then mixed with 20 g of agar and poured to a depth of 10 mm into petri dishes. When set, the cylinders were cut using a 9-mm diameter cork borer and stored at −10 °C. The number of cylinders that were attacked was monitored every 10 min during a period of 1 h. Differences among the number of each type of cylinders destroyed within 10, 30 and 60 min were tested for goodness-of-fit to equal expected numbers.

The second experiment presented individually maintained *C. maenas* of 50–60 mm CW with models made of mussel and oyster shells 15–20 mm in shell length (maximum linear dimension of the shell) filled with either mussel or oyster gel. Intact bivalves were briefly immersed in boiling water to remove all the flesh and thoroughly clean the shells. Empty shells were then dried, and a hole was drilled through the valves so that these could be closed together with fuse wire. Rectangular pieces of gel cut using scalpel were carefully held between the valves, which were then pressed and tied together. Five individually maintained crabs were offered 5 mussel shells + mussel gel and 5 mussel shells + oyster gel; a further five crabs were offered 5 oyster shells + mussel gel and 5 oyster shells + oyster gel. Each time a crab opened a model, the model was replaced by another of the same type to maintain constant availability. The time that elapsed from the moment a crab grasped a model, until that model, whether it was intact or not, was finally abandoned was recorded with a stopwatch, and defined as persistence time. The number of models that were accepted and rejected within 1 h was recorded. The total number of times that each crab encountered each model type (i.e., models with similar shells but different gel) was then tested for goodness-of-fit to the expected values (assuming an equal probability of encounter). Differences in the total number of models that were accepted by each individual crab were tested using the same procedure. Comparisons of persistence time between models of similar shell types but different gel contents were examined using 2-sample *t*-tests on previously log-transformed data.

To assess the importance of shell shape on crab feeding preferences, experiments were carried out by presenting medium and large *C. maenas* with inedible models of comparable size and weight to those of the preferred live prey (Table 1). Models of zero profitability were constructed by filling empty shells of the preferred size range of each prey species with epoxy resin. Models were left 24 h in sea water to eliminate the smell of resin. The combinations of models offered were the same as for the live prey experiments. Trials consisted of presenting four *C. maenas* (50–60 mm CW), each maintained separately, with a sequence of six models of the same prey species. As soon as a crab finished manipulating one model in the sequence, it was presented with the next. On day 1, two crabs were presented with six models of a certain species, and two crabs were presented with six models of another. On day 2, the treatment was reversed so that each crab was presented with a paired combination of model prey (*M–O*, *M–C*, *O–C*, and *M–E*) Persistence time with each model was recorded with a stopwatch and data were logarithmically transformed before analysis of variance using a balanced design with "model in sequence" and "model type" as fixed factors, and "crab" as a random factor.

The importance of shell shape in determining crab persistence was further examined by presenting *C. maenas* of 50–60 mm CW with epoxy resin models of three contrasting geometric shapes: a "cockle" (sphere: 904 mm^3), a "mussel" (wedged rectangle: 420 mm^3), and an "oyster" (flat disc: 530 mm^3; Fig. 1). The resin models were similar in length (maximum linear dimension, all: 12–15 mm) but differed in their height (maximum linear dimension of the axis at right angles to their length: sphere: 12 mm;

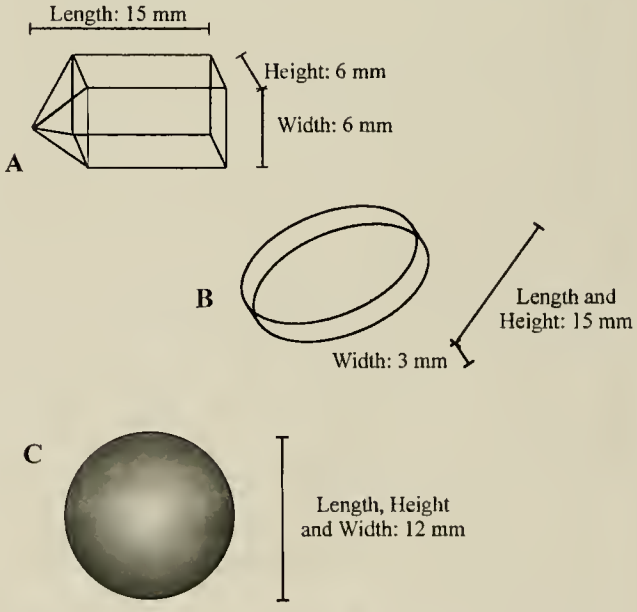

Figure 1. Epoxy resin models of contrasting geometric shapes that were offered to *Carcinus maenas* (50–60 mm CW) to examine the importance of shell shape in crab forging behavior: A) wedged rectangle (420 mm^3), B) flat disc (530 mm^3); C) sphere (904 mm^3).

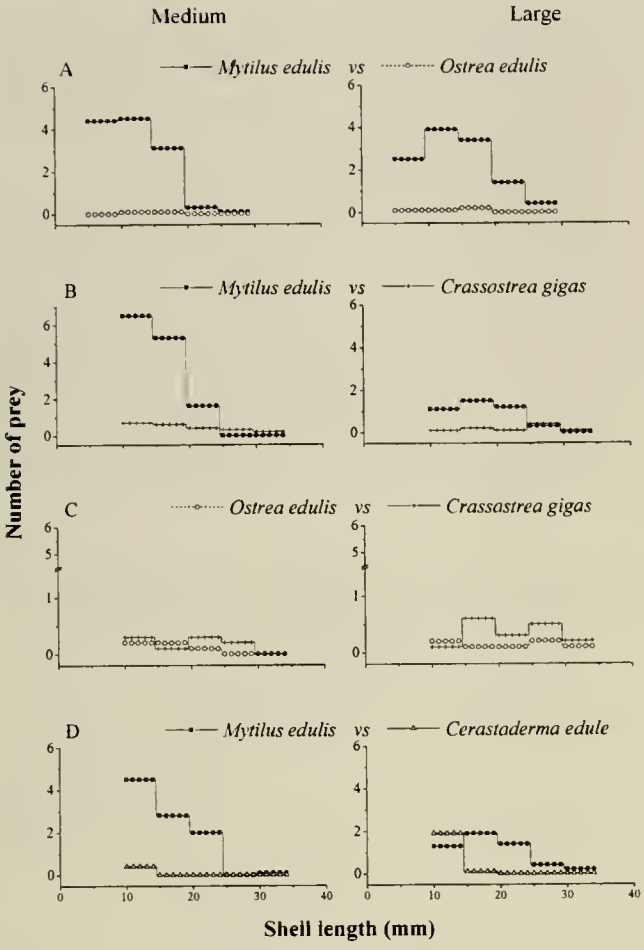

Figure 2. Number of prey of various size classes that were consumed by medium (40–55 mm CW) and large (55–70 mm CW) *Carcinus maenas* during experiments where crabs were offered prey species in paired combinations: (A) *M. edulis–O. edulis*, (B) *M. edulis–C. gigas*, (C) *O. edulis–C. gigas*, (D) *M. edulis–C. edule*. Values are mean consumption rates • $crab^{-1}$ day^{-1}. Note change in scale in (C).

rectangle: 6 mm; disc: 15 mm) and width (minimum linear dimension: sphere: 12 mm; rectangle: 6 mm; disc: 3 mm). On each day, six individually maintained crabs received a sequence of five models of one of the three model types and their persistence times with these models were recorded. Over a 3- d period, each crab had experienced each of the different model types. After each trial, crabs were fed on mussel flesh for 1 h before being starved until the following day. The order in which each crab experienced the different model types was random. Data were logarithmically transformed before differences in persistence time were examined by analysis of variance (ANOVA) using a balanced design with "model in sequence" and "model type" as fixed factors and "crab" as a random factor.

RESULTS

When *M. edulis* was offered in combination with either *O. edulis* or *C. gigas*, both medium (40–55 mm CW) and large (55–70 mm CW) *C. maenas* showed a strong preference for mussels (Fig. 2a,b). When offered a choice between *O. edulis* and *C. gigas*, neither size group of crabs showed any preference for either oyster species (Fig. 2c). When given a choice between *M. edulis* and *C. edule*, medium-sized *C. maenas* clearly preferred mussels; whereas, large crabs consumed similar numbers of both prey of 10–15 mm long, but only consumed mussels from the larger size classes (Fig. 2d).

When crabs were presented with both equal and unequal numbers of the preferred size ranges of *M. edulis–O. edulis*, the percentage of mussels opened by medium and large *C. maenas* (77%–100%) was always significantly higher than that of oysters (Table 2). By contrast, the proportion of *O. edulis* accepted by crabs was never greater than 23%, even when the alternative species was scarce. Of all the mussels encountered, very few were rejected; whereas, any encountered *O. edulis* were only occasionally consumed, and were frequently rejected before the crabs had attempted to open them. Although the percentage of rejected oysters was always high, the already low percentage of rejected mussels in the 1:2 ratio experiments decreased even further in the 1:4 ratio experiments, where mussels were at their lowest relative abundance (Table 2). Results of goodness-of fit tests showed that the number of observed encounters was not significantly different from those expected in all mussel-oyster combination trials (χ^2 from 0.22–2.84, all at $P > 0.05$), suggesting that consumption rates were not influenced by prey encounter rates.

In experiments with equal and unequal numbers of the preferred size ranges of *M. edulis–C. edule*, however, the percentage of accepted and rejected prey varied according to the rates in which prey species were encountered (Table 2). Of the total number of prey accepted by large crabs, the percentage of *M. edulis* decreased when mussels were less abundant. Similarly, the percentage of accepted *C. edule* increased as their relative abundance

TABLE 2.

Percentage and numbers (in parenthesis) of *Mytilus edulis*, *Ostrea edulis*, and *Cerastaderma edule* that were accepted or rejected by *Carcinus maenas* 40–55 and 55–70 mm CW in experiments where crabs were presented with the preferred size classes of mussels and oysters and mussels and cockles in proportions of 1:1, 1:2, and 1:4.

		M. edulis	*O. edulis*	*M. edulis*	*C. edule*
Crab Size	**Outcome**	**1:1**		**1:1**	
40–55 mm	acc	88 (7)*	13 (1)	70 (8)*	30 (3.4)
	rej	36 (3.6)	64 (6.4)	0 (0)	100 (0.4)
55–70 mm	acc	88 (7.4)*	12 (1)	50 (2.6)^{ns}	50 (2.6)
	rej	6 (0.4)	94 (6)	44 (0.8)	56 (1)
		1:2		**1:2**	
40–55 mm	acc	91 (11.4)*	19 (2.6)	54 (8.4)*	46 (7.2)
	rej	4 (0.8)	96 (17.8)	10 (0.2)	90 (1.8)
55–70 mm	acc	100 (2)^{NTA}	0 (0)	38 (4.2)^{ns}	62 (6.8)
	rej	33 (3.8)	67 (7.8)	50 (0.8)	50 (0.8)
		1:4		**1:4**	
40–55 mm	acc	77 (4.6)*	23 (1.4)	49 (8.2)*	51 (8.6)
	rej	4 (0.6)	96 (15.4)	8 (0.4)	92 (4.6)
55–70 mm	acc	100 (1.4)^{NTA}	0 (0)	9 (0.6)^{ns}	91 (6.2)
	rej	11 (0.6)	89 (4.8)	56 (1)	44 (0.8)

Values are mean consumption rates per crab over 1-h periods during 4–5 consecutive days: * denotes prey species accepted in significantly higher numbers than expected ($P < 0.01$); NTA = cases in which results did not allow for chi-square tests to be applied; ns = no significant differences; acc = accepted; rej = rejected.

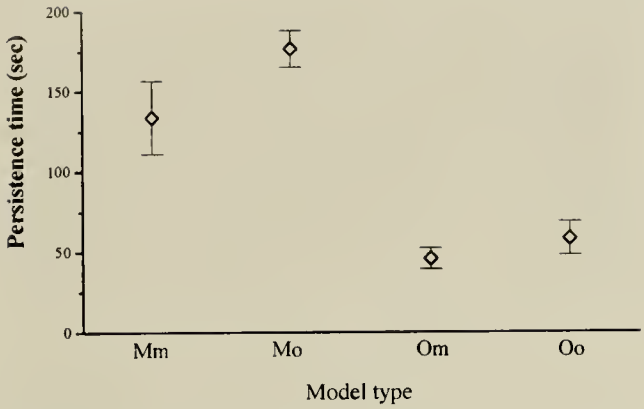

Figure 3. Mean persistence time (sec ± se) taken by *Carcinus maenas* (50–60 mm CW) to open shells of either *Mytilus edulis* (M) or *Ostrea edulis* (O) filled with gels made from either mussel (m) or oyster (o) filtrate.

increased. Large crabs encountered both prey species as expected by the proportions in which they were presented (χ^2 from 0.03–1.63, all at $P > 0.05$). Although medium-sized crabs also accepted mussels and cockles in the same proportions as they encountered them, the encounter rates of mussels and cockles did not correspond to the relative abundance in which prey were offered (1:1 trial: $\chi^2 = 7.48$, $P < 0.05$; 1:2 trial: $\chi^2 = 9.6$, $P < 0.01$ and 1:4 trial: $\chi^2 = 25.77$, $P < 0.001$, respectively). Medium-sized crabs encountered mussels and cockles in statistically indistinguishable numbers in the 1:2 ratio trial ($\chi^2 = 0.05$, $P > 0.05$), and encountered mussels at approximately half the rate that they encountered cockles in the 1:4 ratio trial ($\chi^2 = 1.85$, $P > 0.05$).

When *C. maenas* (50–60 mm CW) were offered a choice between mussel and oyster agar cylinders, these were readily accepted whenever they were encountered. No significant differences in the number of mussel and oyster agar cylinders accepted after 10 min, 30 min, and 1 h were detected (χ^2 from 0.08–0.89, all at $P > 0.05$). When crabs were presented with mussel shells filled with either mussel or oyster gel, they encountered and accepted both model types in similar proportions (encountered: $\chi^2 = 0.31$; $P > 0.05$; accepted: $\chi^2 = 0.20$; $P > 0.05$). When oyster shells filled with either mussel or oyster gel were offered, the encounter rates for both model types were again similar ($\chi^2 = 2.25$; $P > 0.05$), and crabs again accepted similar numbers of oyster shells filled with either type of gel ($\chi^2 = 0.15$; $P > 0.05$). Because crabs showed no apparent preference for one type of gel over the other, results from experiments with different types of gel but similar types of shell were combined. Comparisons of results among experiments with mussel shells and those with oyster shells showed that, although crabs encountered significantly more oyster shells than mussel shells ($\chi^2 = 16.46$; $P < 0.001$), they accepted mussels and oysters in similar proportions ($\chi^2 = 1.13$; $P > 0.05$). Crabs, however, rejected a significantly higher proportion of oyster shells than mussel shells ($\chi^2 = 26.28$; $P < 0.001$), so that in experiments with oyster shells, crabs actually rejected most of the models encountered.

Crabs persisted for similar periods of time with mussel shells filled with either mussel or oyster gel (133.9 ± 21.5 and 176.5 ± 10.8 sec, respectively; $t = -1.84$; $P > 0.05$; Fig. 3). Persistence times were also similar amongst oyster shells filled with either mussel or oyster gel (45.9 ± 6.5 and 58.4 ± 10.2 sec, respectively; $t = -1.01$; $P > 0.05$). Because persistence times with different gel types were in each case similar, results for similar types of shell were combined. Persistence times for mussel shells were significantly longer than for oyster shells regardless of the type of gel with which they had been filled ($t = 7.79$, $P < 0.001$). In summary, our results indicate that prey choice is based on shell shape and strength rather than flesh odour.

When offered five epoxy resin models in sequence, *C. maenas* (50–60 mm CW) persisted for significantly shorter periods of time through the sequence of the first to the last model in all species presentations (*M–O*: $F = 6.78$; $P < 0.001$; *M–C*: $F = 47.16$; $P < 0.001$; *O–C*: $F = 13.23$; $P < 0.001$; *M–E*: $F = 6.31$; $P < 0.001$; Table 3; Fig. 4). Crabs persisted significantly longer with the first model of *M. edulis* presented than with either the *O. edulis* or *C. gigas* models in the *M–O* and *M–C* combinations ($F = 131.76$; $P < 0.001$; $F = 104.60$; $P < 0.001$, respectively), and persisted significantly longer with the first *C. gigas* than with the first *O. edulis* in the *O–C* combination ($F = 18.53$; $P < 0.001$). However,

TABLE 3.

Results of ANOVA for the sequential presentation of epoxy resin models of prey species in four combinations to *Carcinus maenas* of 50–60 mm CW.

Mytilus edulis–Ostrea edulis

Source	*df*	SS	MS	*F*	*P*
Sequence	5	1.94387	0.38877	6.78	<0.001
Model type	1	7.55544	7.55544	131.76	<0.001
Sequence · model type	5	0.38005	0.07601	1.33	0.278
Crab	3	0.32239	0.10746	1.87	0.153
Error	33	1.89235	0.05734		
Total	47	12.09411			

Mytilus edulis–Crassostrea gigas

Source	*df*	SS	MS	*F*	*P*
Sequence	5	6.79175	1.35	47.16	<0.001
Model type	1	3.01310	3.01310	104.6	<0.001
Sequence · model type	5	0.08968	0.01794	0.62	0.683
Crab	3	0.31961	0.10654	3.70	0.021
Error	33	0.95057	0.02881		
Total	47	11.16471			

Ostrea edulis–Crassostrea gigas

Source	*df*	SS	MS	*F*	*P*
Sequence	5	3.23497	0.64699	13.23	<0.001
Model type	1	0.90604	0.90604	18.53	<0.001
Sequence · model type	5	0.19873	0.03975	0.81	0.549
Crab	3	2.84772	0.94924	19.41	<0.001
Error	33	1.61388	0.04891		
Total	47	8.80136			

Mytilus edulis–Cerastoderma edule

Source	*df*	SS	MS	*F*	*P*
Sequence	5	3.1709	0.6342	6.31	<0.001
Model type	1	0.0004	0.0004	0.00	0.951
Sequence · Model type	5	0.2725	0.0545	0.54	0.743
Crab	3	0.9271	0.3090	3.07	0.041
Error	33	3.3184	0.1006		
Total	47	7.6893			

ANOVA: Persistence time = sequence · model type (fixed factors); crab (random factor).

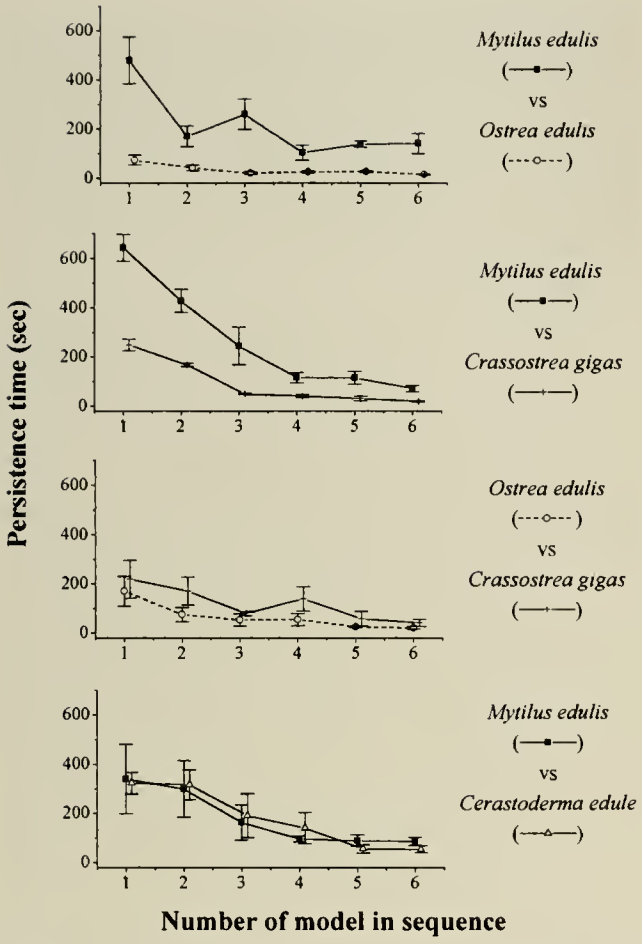

Figure 4. Mean persistence time (sec ± se) for inedible models of *Mytilus edulis*, *Ostrea edulis*, *Crassostrea gigas*, and *Cerastoderma edule* when presented sequentially to *Carcinus maenas* of 50–60 mm CW.

crabs did not differ in their persistence times with the first models of either type in the *M–E* combination ($F < 0.01$; $P > 0.05$). The lack of any significant contribution of the interaction between the model in sequence and model type to the over-all variance of the data for all prey combinations (F from 0.54–1.87; $P > 0.05$) indicates that the decrease in persistence times throughout the sequence of presentation was similar regardless of the type of prey handled by the crabs. Significant differences in the persistence time between individual crabs in the *M–C* ($F = 3.70$; $P < 0.05$), the *O–C* ($F = 19.41$; $P < 0.001$), and the *M–E* ($F = 3.07$; $P < 0.05$) combinations indicates that some of the variability in persistence time is attributable to unexplained differences among individual crabs. However, no significant differences were detected among the crabs in the *M–O* combination ($F = 1.87$; $P > 0.05$; Table 3).

When *C. maenas* (50–60 mm CW) were presented with epoxy resin models of three contrasting geometric shape, persistence time decreased significantly through the sequence of models regardless of their shape (Fig. 5; $F = 20.46$; $P < 0.001$). Although the decrease in persistence time was similar for all three geometric shapes ($F = 0.75$; $P > 0.05$), crabs persisted for a significantly longer period of time with the first sphere and wedged rectangle than with first flat disc ($F = 7.31$; $P < 0.001$). No significant differences in persistence time among individual crabs were detected ($F = 2.00$; $P > 0.05$).

Taken overall, the results of the experiments with epoxy resin models indicate that crabs initially preferred those with a larger minimum dimension (i.e., models of mussels and cockles, and models in the shape of a sphere and a wedged rectangle). Persistence time, however, decreased with increasing number of models in the sequence in a similar rate regardless of model type, suggesting that shell shape does not influence the rate at which preference declines when crabs handle food items of zero profitability.

DISCUSSION

Many studies have related crab preference to the selection of prey with a higher value or profitability, expressed as the net energy intake per unit of handling time (Elner and Hughes 1978, Elner and Raffaelli 1980). However, authors have noted that selection of optimum prey could result from a greater chance of encountering items with a larger surface area and/or reduced handling times because of a lower predatory resistance (Barbeau and Scheibling 1994), in which case, prey selection should be considered to be a consequence of passive choice. Passive or mechanistic prey selection usually results from physical properties and behavior of both predator and prey (Hughes 1980) that determine the probability of encountering and opening alternative prey types. Body size, speed of movement, and density of predator and prey can influence the probability of encountering, recognizing, and predicting valuable prey (Lawton 1989); whereas, contrasting morphologies in bivalve shells and the mechanical properties involved in crab attack can affect prey vulnerability (Boulding 1984, Brown and Haight 1992), and hence the probability of opening encountered prey (Sih and Moore, 1990).

When crabs were offered a wide size range of oysters and mussels simultaneously, both medium (40–55 mm CW) and large (55–70 mm CW) crabs consistently selected mussels (Fig. 2). Furthermore, results of experiments with *M. edulis* and *O. edulis* in equal and altered proportions suggest that preference for mussels, and the apparent lack of preference for oysters, are independent of the relative abundance in which either prey species are presented (Table 2). Previous authors have shown that *C. maenas* consumed *M. edulis* at more than twice the rate at which they consumed *C. gigas* (Dare *et al.* 1983); whereas, *Cancer novaezelandiae* also preferred mussels when offered a choice of mussels and gastropods (Creswell and McLay, 1990).

Barbeau and Scheibling (1994) indicated that active selection can be considered to be an important component of predation when a predator selects a prey type more often than expected when given a choice of prey types than when not given a choice (see also Liszka and Underwood 1990). In our study, comparisons of prey consumption rates in single and multiple choice experiments could not be made, thus, active and passive components could not be analyzed in this way. However, the preference for *M. edulis* exhibited by *C. maenas* was consistent throughout experiments where prey types were encountered in varying and contrasting proportions (Fig. 2; Table 2). In earlier single species experiments involving *M. edulis*, *O. edulis*, *C. gigas*, and *C. edule* designed to establish whether prey selection in *C. maenas* was size related, the order in which prey species were ranked according to consumption rates clearly paralleled the rank order of species profitability (Mascaró and Seed 2000). We reported that differences in profitability between prey species were mainly attributable to differences in their biomass, rather than to differences in breaking time. These results suggest that prey value can influence prey species-selection

and that crab preference for mussels in the present study involves an active component of selection at some point of the predation cycle.

When *C. maenas* were offered *M. edulis* and *C. edule* in various proportions, species selection was strongly influenced by the frequency in which each size category of crabs encountered prey (Table 2). Although active selection could not be invalidated (*sensu* Barbeau and Scheibling 1994), the close agreement in the proportions of accepted and encountered prey items suggests that the active component of selection in this particular prey combination is not important in determining crab preference. The observed differences in the foraging behavior of *C. maenas* when feeding on a combination of mussels and oysters and a combination of mussels and cockles further supports the view that the relative importance of active and passive selection in explaining prey choice may differ with each predator–prey system (Abele *et al.* 1981).

For active prey selection, mechanisms must exist by which crabs are able to recognize prey characteristics that correlate with their potential value. Among prey near the optimal size, such characteristics as shell shape or flesh odor/flavor may have an important effect on foraging decisions. Amino acids can be readily differentiated by *C. maenas* during searching (Shelton and Mackie 1971) and feeding phases (Case and Gwilliam 1961). Our results show that crabs were not attracted any more often to gel cylinders made from mussel-flesh filtrate than to those containing oyster-flesh filtrate. Crabs attacked both types of cylinders whenever these were encountered and this behavior persisted throughout the duration of the feeding trials, suggesting that preference for either type of flavor was not modified once crabs had experienced the gels.

A study of the factors affecting diet selection in *C. maenas* demonstrated that shore-crabs are sensitive to different concentrations of mussel flesh filtrate, and suggested that, despite dilution effects, crabs may be able to distinguish mussels of varying quality by responding to odor (Kaiser *et al.* 1993). In addition, these authors indicated that olfactory stimuli had a reinforcing effect on crab preference, because crabs readily picked up and manipulated both models with and without mussel filtrate, but more quickly rejected those that did not incorporate the chemical stimulus. Our experiments did not include any examination of different concentrations of flesh filtrate, but mussel and oyster flesh filtrates had a similar reinforcing effect on the perseverance with prey, and crabs initially attacked either type of model irrespective of their flavor (Fig. 3). These results provide further evidence of the reinforcing effect of olfactory and taste stimuli, and comparisons with previous reports suggest that perhaps the concentration, rather than the specific chemical composition, of flesh might be involved in prey attractiveness to crabs (Shelton and Mackie 1971). Our results do, however, suggest that certain characteristics of the shells of *M. edulis* and *O. edulis* might provide mechanical stimuli that influence decisions by crabs initially to attack and to persist with prey that is already being manipulated (Fig. 3).

When attacking hard-bodied, resistant prey, predatory crabs show relatively stereotyped patterns of behavior (Lau 1987, Seed 1993), but details of this behavior can be influenced by specific morphological characteristics among prey of different size (Elner and Hughes 1978). Over a relatively narrow range of prey size; however, shape can have a marked influence on the handling process (Boulding 1984, Griffiths and Seiderer 1980), and information gained by crabs while rotating prey with the chelae and mouth parts can be crucial in deciding whether or not the attack is continued or aborted (Akumfi and Hughes 1987). In experiments where *C. maenas* were offered a sequence of five models of each prey species, crabs persisted longer with the first model of a mussel to be offered than with the first model of an oyster, and they also persisted longer with the first model of *C. gigas* than with *O. edulis* (Table 3; Fig. 4). These results suggest that the initial reluctance of crabs to attack flat oysters and their propensity to attack mussels, are not associated with differences in the ultimate energetic reward, but could be based on an evaluation of the overall shape and/or volume of the shells of these bivalves during a first recognition phase.

Further investigation of the importance of shell shape in species selection showed that *C. maenas* persisted much longer with the first models that resembled both the shape of a cockle (sphere) and a mussel (wedged rectangle) than with the first model that resembled a flat oyster (disc; Fig. 5). The resin models used had a similar length, but differed in their height and width, the flat disc having the smallest width of the three models (Fig. 1). If crabs evaluate prey on the basis of shell width rather than length, they would be expected to persist longer on those shells having the greatest width. A strong association between shell width and volume is suggested by the significantly greater increase in shell width with increasing shell length in cockles and mussels, compared to flat oysters, and by flat oysters having significantly less flesh weight than mussels and cockles of comparable shell length (Mascaró and Seed 2000). Furthermore, *C. maenas* obtained the greatest profitability when feeding on cockles and mussels (Mascaró and Seed 2000), and *C. edule* and *M. edulis* were included in crab diets more frequently than *O. edulis* throughout the species-selection experiments (Table 2, Fig. 2). These results suggest that shell width; that is, the minimum linear shell dimension, probably constitutes an important morphological characteristic that crabs are able to evaluate and associate with potential prey value.

Previous studies have reported that the minimum shell dimension is an important characteristic determining prey size and species selection in crabs (Boulding 1984) and in other decapods (Griffiths and Seiderer 1980). In experiments where *C. maenas* was presented with Perspex models of different shape and size, Kaiser *et al.* (1993) found that changes in model length had little influence on handling time, because the mechanical efficiency of the chelae was determined by the cross-sectional profile of the prey. These authors further suggested that those models that more closely resembled the shape of a mussel (wedged rectangle) allowed the chelae to operate at maximum mechanical advantage and improved handling efficiency. Mascaró and Seed (2000) re-

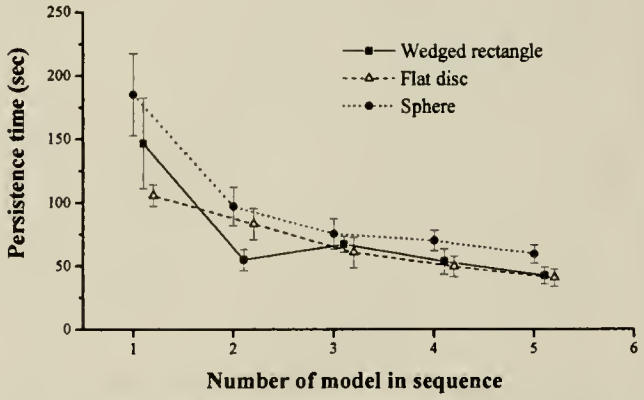

Figure 5. Mean persistence time (sec ± se) for inedible models of three contrasting geometric shapes (wedged rectangle, flat disc and sphere) that were presented sequentially to *Carcinus maenas* of 50–60 mm CW).

ported that the relative prey size (= shell width/maximum cross-sectional dimension of the major chela) preferred by *C. maenas* consistently had values <1, suggesting that the position in which the width of the shell fitted within the chela was significant in determining attack success of the preferred size classes of prey. In the present study, flat oysters were more difficult to accommodate within the chela than were mussels. Although flat oyster shells are very narrow, their large shell length and height reduce their handling efficiency. In contrast, mussel shells with only one of its linear dimensions being large, can be accommodated within the chela either with its height or its width in the gape between propus and dactylus.

If foraging decisions by crabs were based on shell width, then some patterns of species selection by *C. maenas* observed in this study could be explained. The observed similarity in consumption rates of *M. edulis* and *C. edule* might be attributable to the similarity in shell width of the (different) size classes of these prey offered (Table 1). When crabs were offered similar size classes of *M. edulis* and *O. edulis*, they preferentially consumed mussels probably because of the differences in shell width between the (similar) size classes of prey presented (Table 1).

Although some portunids can use vision to locate their prey (Hughes and Seed 1981, Seed and Hughes 1997), crabs are essentially nonvisual predators. This makes olfactory stimuli the predominant factor directing crabs toward potential prey (Barber 1961). Although different concentrations of chemical compounds elicit different components of the searching response (i.e., locomotory or probing and grasping reflexes; Zimmer-Faust and Case 1982), our results suggest that during this initial phase, differences in flesh odor/flavor between different prey species did not seem to influence prey selection. Once crabs have physically encountered a prey item, shape and size of the shell assume a greater significance than flesh odor, interacting with chemical and mechanical stimuli from alternative prey touched by the pereiopods (Jubb *et al.* 1983). Our results emphasize the importance of certain shell characteristics and suggest that those dimensions more strongly associated with prey volume (e.g., shell width), and, hence, the amount of potential flesh, might be predominant in deciding whether a crab attacks. Once a food item has been recognized and an attack initiated, further decisions can be influenced by several factors, including microfractures within the shell structure (Boulding and LaBarbera 1986) and leakage of body fluids that stimulate crabs to persist with the attack. Stimuli from alternative prey may also deter crabs in their opening attempts. Moreover, it is at this stage, when a prey item that does not yield easily to the crab's crushing efforts can be abandoned. Thus, prey vulnerability, together with the tenacity and experience of crabs might be particularly relevant at this point of the encounter. Although our results do not allow a hierarchical order of crab responsiveness to various prey stimuli to be defined, they do emphasize that odor and shape assume different importance at several decisive moments of the foraging bout, and that by using a variety of stimuli to assess prey quality, crabs probably enhance their predatory efficiency.

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THE EFFECTS OF HYPERCAPNIC HYPOXIA ON THE SURVIVAL OF SHRIMP CHALLENGED WITH *VIBRIO PARAHAEMOLYTICUS*

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ABSTRACT Estuarine organisms routinely encounter fluctuations in dissolved oxygen, carbon dioxide, and pH, which can vary both seasonally and diurnally. Such environmental stresses as hypoxia can affect the immune response of invertebrates and vertebrates and have been linked to increased disease incidence. This research investigated the effects of hypoxia, hypercapnia, and low pH on disease susceptibility in both penaeid and palaemonid shrimp. Juvenile penaeid shrimp *Litopenaeus vannamei* and adult grass shrimp *Palaemonetes pugio* were challenged by intramuscular injection with a previously determined LD_{50} dose of a known pathogenic strain of *Vibrio parahaemolyticus*. Mortalities were monitored for shrimp held under normoxia (Po_2 = 150–155 torr, Pco_2 = 0.23 torr, pH = 7.6–8.0 for *L. vannamei*, Po_2 = 150–155 torr, Pco_2 = 0.23 torr, pH = 8.0–8.2 for *P. pugio*) and two levels of hypoxia. The penaeid shrimp were challenged under normocapnic hypoxia (Po_2 = 45 torr, Pco_2 = 0.23 torr, pH = 7.8–8.1) and hypercapnic hypoxia (Po_2 = 30 torr, Pco_2 = 15.2 torr, pH = 6.8–7.0). Grass shrimp were challenged under two levels of hypercapnic hypoxia (Po_2 = 45 torr and 30 torr, Pco_2 = 15.2 torr, pH = 6.7–7.0). Both the juvenile *L. vannamei* and the adult *P. pugio* held under hypercapnic hypoxia at 30 torr oxygen displayed significantly lower 48-hour survival (15.7 and 3.1%, respectively) than animals held in normoxic water (28.7 and 29.4%, respectively). There was no significant decrease in survival in *L. vannamei* under normocapnic hypoxia at 45 torr oxygen or in *P. pugio* under hypercapnic hypoxia at 45 torr oxygen. Total hemocyte count (THC/mL) significantly decreased in adult *L. vannamei* held under hypercapnic hypoxia when compared to normoxic controls. Oxygen level had a significant effect on total hemocyte density; whereas, time and the interaction of time and oxygen did not. The reduction in THC/mL may contribute to an increased rate of mortality in shrimp held under hypoxic conditions and challenged with *V. parahaemolyticus*. These results show that hypercapnic hypoxia decreases survival following bacterial challenge in both *L. vannamei* and *P. pugio* and decreases total hemocyte count in *L. vannamei*. These data provide direct evidence that naturally occurring variations in oxygen, CO_2 and pH can place estuarine organisms at increased risk from opportunistic pathogens

KEY WORDS: LD_{50}, hypercapnia, hypoxia, palaemonid, penaeid, shrimp, *Vibro*

INTRODUCTION

Penaeid and Palaemonid shrimp in estuarine waters frequently encounter levels of dissolved oxygen, carbon dioxide, and pH that vary dramatically on a diurnal and seasonal basis. Shallow coastal regions in the southeast and in the Gulf of Mexico often experience dissolved oxygen concentrations less than 3.0 mg/L (Breitburg 1990, Rabalais et al. 1994, Burnett 1997, Summers et al. 1997). In South Carolina tidal marshes, tidal creek oxygen pressures can fluctuate between 9 and 170 torr (6% and 110% air saturation) within a 24-hour period (Cochran and Burnett 1996). Oxygen levels as low as 1.2% air saturation (approximately 2 torr) have been measured in the nearby Savannah River estuary (Winn and Knott 1992). Moreover, hypoxia is almost always accompanied by an increase in carbon dioxide pressure (Pco_2), or hypercapnia, produced by respiration. Elevated levels of water CO_2 then drive a decrease in water pH. Cochran and Burnett (1996) reported that Pco_2 varies from 0.3 to 12 torr, and pH ranges from 6.5 to 7.6 in South Carolina tidal marshes. Thus, hypoxia and low pH often co-occur in the natural environment (Burnett 1997).

Shrimp raised in aquaculture ponds also experience severe changes in O_2, CO_2, and pH because of high density and nutrient input from feed (Browdy et al. in press, Madenjian 1990). Dissolved oxygen levels are routinely measured in well-managed farm ponds, with the general understanding that low O_2 levels may be lethal to shrimp. Supplemental aeration is used to reduce fluctuations in dissolved oxygen; however, periods of hypoxia and hypercapnia still occur in routine management (Chang and Ouyang 1988, Garcia and Brune 1991).

Although extreme hypoxia or anoxia can cause mass mortalities in estuarine organisms (Garlo et al. 1979, Winn and Knott 1992, Diaz and Rosenberg 1995, Lenihan and Peterson 1998), sublethal hypoxia may have an adverse impact on normal physiological functions in shrimp, such as osmoregulatory capacity (Charmantier et al. 1994) and molting (Clark 1986). Hypoxia also can suppress immune function in shrimp. Direkbusarakom and Danayadol (1998) found that hypoxia (1.8–2 ppm) decreased phagocytosis and bacterial clearance efficiency in the black tiger shrimp, *P. monodon*. In addition, Le Moullac et al. (1999) reported a decrease in hemocyte numbers and respiratory burst activity of *Litopenaeus stylirostris* exposed to severe hypoxia (1 mg O_2/L). These observations suggest that chronic sublethal hypoxia might suppress the ability of shrimp to resist infections with opportunis-

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tic pathogens such as environmental bacteria, viruses, and fungi. Indeed, Le Moullac (1999) demonstrated that the levels of hypoxia that decreased hemocyte numbers and suppressed respiratory burst activity in *L. stylirostris* also increased pathogenicity of *Vibrio alginolyticus* in that shrimp species.

Unfortunately, most studies of hypoxia ignore changes in CO_2 and pH associated with hypercapnic hypoxia (Burnett 1997). Where the effects have been assessed, low pH and high CO_2 enhanced mortality rates of extreme hypoxia (Martinez et al. 1998) and altered metabolic activity (McCulloch 1990). Low pH independently and additively with hypoxia suppressed the respiratory burst of oyster hemocytes (Boyd and Burnett 1999). The latter study strongly suggested that hypercapnic hypoxia suppresses the resistance of wild and aquacultured estuarine organisms against such naturally occurring opportunistic pathogens as bacteria, viruses, and fungi.

In the last 10 years, several highly lethal bacterial pathogens have had a serious impact on both wild and aquacultured populations of penaeid shrimp (Karunasagar et al. 1994, Mohney et al. 1994, Hiney 1995, Liu et al. 1996, Alapide-Tendencia and Dureza 1997, Lavilla-Pitogo et al. 1998). The most frequently reported bacterial infection in penaeid shrimp is vibriosis, caused by bacteria from the family Vibrionaceae (Adams 1991, Sahul Hameed 1995). Bacteria in the family Vibrionaceae comprise 10–50% of the marine heterotrophic bacteria found in coastal waters (Thune et al. 1993). Among several *Vibrio* species associated with this disease, *Vibrio parahaemolyticus* is frequently associated with disease outbreaks in aquaculture (Mohney et al. 1994, Sahul Hameed 1995) and is sometimes found at high densities in coastal waters (Buck 1990, DePaola et al. 1990).

In this study, we evaluated the impact of hypoxia and hypercapnic hypoxia on resistance to the opportunistic bacterial pathogen *V. parahaemolyticus* in two commercially and recreationally important species of shrimp. The Pacific white shrimp, *Litopenaeus vannamei,* is the species of choice for penaeid shrimp aquaculture in South Carolina. *L. vannamei* occurs naturally from the Gulf of California to northern Peru (Perez Farfante and Kensley 1997), but is imported for use in aquaculture because of its faster growth over native species (Sandifer et al. 1993). The grass shrimp, *Palaemonetes pugio,* serves an important role in the estuary as a detritivore by consuming and breaking down *Spartina* and aiding in trophic level energy transfer (Welsh 1975). They also serve as prey for many important commercial and recreational fishes and crustaceans, which use the marsh as nursery grounds (Welsh 1975). In the first phase of these experiments, an intramuscular bacterial challenge model with survival endpoint was developed and applied in both shrimp species to determine LD_{50} values for *V. parahaemolyticus.* Then, to evaluate the contribution of hypoxia and hypercapnic hypoxia to disease resistance, survival was monitored in shrimp challenged with LD_{50} doses of *V. parahaemolyticus* and exposed to varying levels of water O_2, CO_2, and pH. Finally, to determine whether hypercapnic hypoxia might alter cellular components of the shrimp immune system over the time course of these bacterial challenges, total hemocyte densities of the hemolymph (THC/mL) were compared in animals exposed to normoxia and hypercapnic hypoxia.

MATERIALS AND METHODS

Experimental Animals

Litopenaeus vannamei (Boone) from specific pathogen-free stocks were provided by the Waddell Mariculture Center in Bluffton, South Carolina, by Island Fresh Seafood in Yonges Island, South Carolina, and by Dixieland Maricultural Farms in Hollywood, South Carolina. Shrimp were maintained in well-aerated recirculating seawater at 28–32 ppt salinity, 23–25 °C, and pH 8.0–8.2. Water quality variables (pH, salinity, and temperature) were measured every other day. Ammonia was monitored twice a month and remained lower than 0.25 mg/L. Animals were fed once daily with shrimp feed (Zeigler Brothers, Inc). All necessary precautions were followed for possessing a nonindigenous shrimp species as outlined in the nonindigenous shrimp possession permit #NI98-0565 granted by the South Carolina Department of Natural Resources.

Grass shrimp *Palaemonetes pugio* (Holthuis) were collected with a dip net in a nearby tidal creek. These shrimp were held in a 50-gallon aquarium at 25–27 ppt salinity and 23–25 °C for at least 2 days before use in an experiment. Animals were fed Marine Tetra Flakes daily.

Bacteria

A known pathogenic strain of *Vibrio parahaemolyticus* (90-69B3) was streaked on a Tryptic Soy Agar (TSA) plate with 2.5% NaCl added and allowed to grow overnight at room temperature. Aliquots (0.5 mL) of the bacteria were stored in freezing media (Tryptic Soy Broth (TSB) + 2.5% NaCl and 20% glycerol) at −70 °C. These aliquots were used as the working stock.

For each assay, *V. parahaemolyticus* was streaked onto TSA + 2.5% NaCl plates from the frozen aliquots and allowed to grow at room temperature for 24 hours before use. A different aliquot was used for each assay to avoid excessive passages of the bacteria on plates. Bacteria were transferred from the plates to 2.5% NaCl buffered with 20 mmol/L HEPES using wooden applicator sticks. Bacterial densities were quantified by optical density (OD) at 540 nm and then serially diluted in the saline to obtain the test dosages. OD values were confirmed by counting colony-forming units on double layer plates (10 mL of marine agar containing the bacterial dilution overlaid onto 10 mL of TCBS agar). OD values of 0.1 and 1.0 were determined to be equal to 1.0×10^8 colony-forming units per mL (CFU/mL) and 1.0×10^9 CFU/mL, respectively. Koch's postulates were satisfied to confirm the pathogenicity and relationship between *V. parahaemolyticus* and vibriosis (Prescott et al. 1996).

The identity of the bacteria used in challenge tests and after each isolation of Koch's postulates was confirmed using Gram strains, motility tests, characterization of growth on TCBS plates, cytochrome oxidase tests, and API-20NE test strips for Gram-negative, nonfermentative bacteria (API resultant bacteria #7276644). Aseptic techniques were used when working with the bacteria. Waste material was either autoclaved or disinfected with 1% chlorine bleach.

LD_{50} Tests for Litopenaeus vannamei

Vibrio parahaemolyticus was streaked onto TSA + 2.5% NaCl plates from the frozen aliquots as described above. Juvenile animals (5.8 to 8.9 cm and weighing from 1.0 to 4.2 g) were injected intramuscularly near the fourth ventral abdominal segment using a Hamilton syringe with 50 µL of bacterial suspension (ranging from 5×10^3 to 5×10^7 CFU/shrimp) or with 2.5% NaCl buffered with 20 mmol/L HEPES without bacteria as a control. Animals were then placed in 3.5 L, wide-mouth, screw-lid, glass jars with 700 mL of filtered (0.45 µm) artificial seawater (ASW) adjusted to 30 ppt. Lids of the test containers were fitted with tubes for in-

coming air and an air release tube (61 cm) with two cotton plugs to contain *Vibrio* aerosol. Seven animals were placed in each jar with three replicates for each dose. LD_{50} tests were performed under normoxic conditions (155 torr oxygen), with low CO_2 (less than 1 torr) and high pH (pH 7.7–7.9), and mortality was recorded at 2, 4, 8, 12, 24, and 48 hours after injection of *Vibrio*. Water was changed in all jars at 12 and 24 hours after feeding and then when necessary in individual jars (i.e., when the water became cloudy because of shrimp mortality). Animals were fed commercial shrimp food (as above) every 12 hours. The *L. vannamei* LD_{50} test was repeated three times. LD_{50} and confidence intervals for both species were calculated using the EPA Trimmed Spearmann–Karber program (Hamilton et al. 1977).

LD_{50} Tests for Palaemonetes pugio

The methods for the *P. pugio* LD_{50} tests were similar to those mentioned above for *L. vannamei*, with a few exceptions. Because *P. pugio* were smaller (2.1 to 3.4 cm and weighing from 0.2 to 0.4 g), only 5 μL of a saline containing bacteria was injected, and the shrimp were held in smaller test chambers with 400 mL of ASW. Water in all experimental jars was changed once every 24 hours. Animals were fed Marine Tetra Flakes (as above) every 12 hours. The *P. pugio* LD_{50} test was repeated two times with different bacterial concentrations for each (ranging from 2.25×10^1 to 2.25×10^5 CFU/shrimp for test 1, and ranging from 5×10^1 to 5×10^5 CFU/shrimp for test 2).

Challenge Test Design

It was not possible to maintain appropriate levels of oxygen in the jars used for the *L. vannamei* and *P. pugio* LD_{50} tests by directly using Wösthoff gas mixing pumps and individually aerating the jars. This was because of the low output of the pump, the variability in aeration to each jar, and the high oxygen demand of the shrimp. Therefore, a new experimental design was employed for the hypoxic challenge tests for both species. Ten-gallon aquaria were divided into four chambers of equal size to hold the shrimp and one smaller chamber to hold a circulating pump (see below) using Plexiglas drilled with holes to allow water to flow freely among the chambers. Nine L of 30 ppt filtered (0.45 μm) artificial seawater (Crystal Sea marine mix) was added to each tank. A small, submersible pump (Penguin 550) in each experimental tank circulated water among the compartments. Normoxia was maintained by vigorous aeration. Hypoxia was maintained by controlling aeration. The consumption of oxygen by the shrimp lowered the oxygen pressure in the water. Oxygen pressure in the water was monitored using an oxygen electrode and meter (YSI Model 58). Output from the oxygen meter was monitored by a Sable System data acquisition system, which was used to control tank aeration by an air stone at a user-defined setpoint. To control water CO_2 pressures, a Wösthoff gas mixing pump delivered mixtures of CO_2 and nitrogen continuously. The CO_2 and N_2 gas mixture also served to lower the oxygen pressure. At steady state, this system maintained constant oxygen and CO_2 pressures (Fig. 1). Oxygen levels in the chambers remained within 1 torr of the set value.

L. vannamei *Challenge Tests at 45 torr O_2–Normocapnic Hypoxia*

L. vannamei challenge tests were performed under two levels of hypoxia mimicking two different environmental conditions (see Table 1). The first set of tests compared disease susceptibility between animals held under normoxia and animals held under normocapnic (i.e., very low CO_2 pressure) hypoxia with no added CO_2 (treatment 1). This test evaluated the effect of low oxygen only. For these tests, ASW was made hypoxic by bubbling pure N_2 into the first chamber. Gassing the water with N_2 drove off excess CO_2, keeping pH and CO_2 at normoxic levels, with pH 7.8–8.1 and 0.03% CO_2. Normoxia was maintained by bubbling ambient air into the tanks through three air stones.

Using the method described above, juvenile shrimp ranging from 5.7 cm to 9.0 cm (1.0 g to 4.5 g) were injected intramuscularly with 50 μL of bacterial suspension or with 2.5% NaCl buffered with 20 mmol/L HEPES. The bacterial numbers for these tests ranged from 1.8×10^6 CFU/shrimp to 2.25×10^6 CFU/shrimp, which were greater than the previously determined average LD_{50} but were within the 95% confidence interval (Table 2). Nine shrimp with or without injected bacteria were placed in each of the four chambers of the appropriate tanks for a total of 36 animals per tank. Animals were placed randomly in either the hypoxic or normoxic tanks. All four treatments (one per tank) were conducted simultaneously and were counted as one replicate. Mortality was recorded, and dead or moribund animals were removed at 2, 4, 8, 12, 16, 20, 24, 28, 32, 36, 40, 44, and 48 hours after injection challenge. Water was changed in all tanks at 12, 24, and 36 hours, and then whenever necessary in individual tanks. The replacement water for the normocapnic hypoxia treatment was gassed ahead of time to appropriate treatment pressures to avoid a change in oxygen pressure. Animals were fed commercial shrimp food every 12 hours. This challenge test was repeated three times.

L. vannamei *Challenge Tests at 30 torr O_2 + 15.2 torr CO_2–Hypercapnic Hypoxia*

The second set of tests (treatment 2) compared disease susceptibility between animals held under normoxia and animals held under hypercapnic hypoxia at 30 torr oxygen and 15.2 torr CO_2 (4 and 2%, respectively). These tests were conducted as described above (treatment 1); however, the CO_2 and pH were adjusted to mimic hypoxic levels (Table 1). The resulting pH of the hypoxic water was 6.8–7.0. The bacterial concentration used in these tests was 1.125×10^6, which was within the 95% confidence interval previously determined in the LD_{50} tests. This challenge test was repeated three times.

P. pugio *Challenge Tests*

Both of the grass shrimp challenge tests compared disease susceptibility between animals held under normoxia and animals held under hypercapnic hypoxia. These challenge tests were performed under two levels of hypercapnic hypoxia: 45 torr O_2 + 15.2 torr (2%) CO_2 and 30 torr O_2 + 15.2 torr (2%) CO_2. Hypercapnic hypoxia was achieved as described above for both treatments with only the set point for the data acquisition system differing between the two levels of hypoxia (Table 1).

The methods for the *P. pugio* challenge treatments were similar to those mentioned above for the *L. vannamei* treatments, with a few exceptions. For the grass shrimp tests, only 5 μL of a saline containing bacteria was injected, and 10 animals were placed in a chamber for a total of 40 animals per tank. The bacterial concentrations for these tests ranged from 9.10×10^4 to 1.25×10^5 CFU/shrimp. These values were outside of the confidence interval previously determined in the LD_{50} tests because of an experimental error in the original calculation of the confidence intervals. However, because the same bacterial concentration was used for both treatments in a test, the results were unaffected. These challenge tests were each repeated four times.

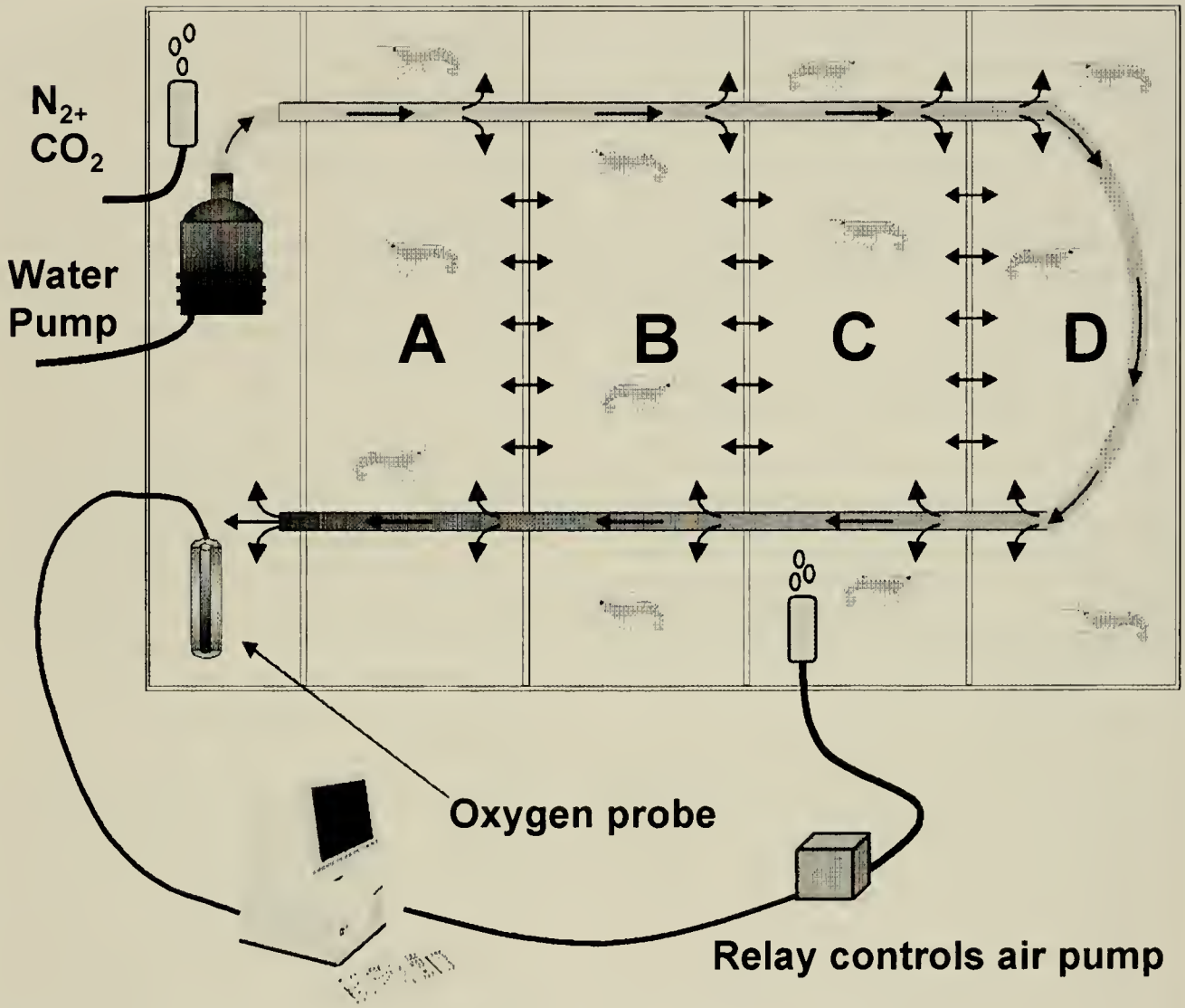

Figure 1. Schematic drawing of the tanks used to hold animals under different gas pressures. Oxygen pressure was measured with an oxygen electrode and adjusted using an air pump controlled by a computer. The infusion of air was opposed by gassing with mixtures of nitrogen and carbon dioxide (depending on the experiment). The tank was divided into compartments, and the water was circulated using a submersible pump.

Statistical Analysis for Challenge Tests

Challenge tests were performed using a full factorial design with bacteria and oxygen as the effect variables. Each challenge test produced four survival curves: normoxia without bacteria, normocapnic hypoxia (hypoxia with very low CO_2) or hypercapnic hypoxia (hypoxia with elevated CO_2) without bacteria (depending on the treatment in question), normoxia plus bacteria and normocapnic hypoxia or hypercapnic hypoxia plus bacteria. Using the statistical program JMP IN (SAS Institute, Inc.), a quadratic polynomial was fitted to each curve to obtain an intercept, response coefficient, and response coefficient^2 for each line. The combined coefficient and coefficient^2 parameters were then analyzed as the response variables in a multivariate analysis of variance (MANOVA) with bacteria, oxygen, and the interaction of bacteria and oxygen (bacteria*oxygen) as the x values. Differences in the coefficient and coefficient^2 revealed differences in the survival rate of shrimp among treatments. The intercepts were not analyzed, because differences in the intercept were artifacts of fitting a quadratic polynomial to a survival curve and did not reveal information about the rate of survival. Four MANOVA tests were run, one for each suite of tests: *L. vannamei* at 45 torr oxygen and <1 torr CO_2, *L. vannamei* at 30 torr oxygen with 15.2 torr CO_2, *P. pugio* at 45 torr oxygen with 15.2 torr CO_2, and *P. pugio* at 30 torr oxygen with 15.2 torr CO_2. Results of the MANOVA tests revealed if oxygen level/CO_2 treatment (normoxia vs. hypoxia), bacteria (absence vs. presence) and/or the interaction of the two (bacteria*oxygen) had a significant effect on ($P < 0.05$) shrimp survival following bacterial challenge. Univariate analysis of variance tests (ANOVAs) were then run to see if the significance found in the MANOVA was attributable to coefficient, coefficient^2, or both.

Total Hemocyte Count

The impact of hypercapnic hypoxia at 30 torr oxygen, 15.2 torr CO_2 and pH 6.9–7.1 on total hemocyte count/mL hemolymph in

TABLE 1.

Water quality variables used in the challenge tests and total hemocyte count (THC) assay.

Vibrio Challenge Tests	O_2			CO_2		
	torr	% air sat.	mg/L	torr	%	pH
Litopenaeus vannamei						
Normoxia (control)	150–155	21	7.29	0.23	0.03	7.6–8.0
Normocapnic hypoxia treatment 1	45	6	2.12	0.23	0.03	7.8–8.1
Hypercapnic hypoxia treatment 2	30	4	1.41	15.2	2	6.8–7.0
Palaemonetes pugio						
Normoxia (control)	155	21	7.29	0.23	0.03	8.0–8.2
Hypercapnic hypoxia treatment 1	45	6	2.12	15.2	2	6.9–7.0
Hypercapnic hypoxia treatment 2	30	4	1.41	15.2	2	6.9–7.0
Total hemocyte count						
Normoxia (control)	150–155	21	7.29	0.23	0.03	8.0–8.2
Hypercapnic hypoxia	30	4	1.41	15.2	2	6.9–7.1
Ranges of variables observed in nature	0–285	0–38.6	0–14	0.23–35.6	0.03–4.7	6.5–8.3

O_2 and CO_2 are presented several ways for comparison with water quality data in the literature. The following references were used to report the environmental ranges listed for O_2, CO_2, and pH: Breitburg, 1990, Winn and Knott, 1992; Rabalais et al., 1994; Cochran and Burnett, 1996.

Litopenaeus vannamei was measured over the 48 hours to replicate the time period of the challenge tests. At time zero, shrimp were placed randomly in normoxic or hypercapnic hypoxia tanks. Hemolymph from individual adult *L. vannamei* was withdrawn from the ventral sinus at the base of the fourth or fifth walking leg at a specified time point (4, 8, 16, 24, or 48 hours) into a 1.0 mL syringe with a 26-gauge needle. Hemolymph was diluted with an anticoagulant solution (AS) described by Lee et al. (1995): 20% filtered seawater, 30 mmol/L trisodium citrate, 0.1 mmol/L glucose, 26 mmol/L citric acid, 10 mmol/L EDTA at pH 4.6. Total hemocyte counts were performed using a hemocytometer, taking into account the dilution of the hemolymph with AS during bleeding. Twenty shrimp were bled at 4, 16, 24, and 48 h (10 each from normoxia and hypercapnic hypoxia); 22 shrimp were bled at 8 h (11 each from normoxia and hypercapnic hypoxia). Individual shrimp were used only once. Mortality was monitored throughout the experiment.

TABLE 2.

48-Hour LD_{50} values for *Litopenaeus vannamei* and *Palaemonetes pugio* for *Vibrio parahaemolyticus*.

Test	48-hour LD_{50}	95% Confidence Interval
Litopenaeus vannamei		
1	6.04×10^5 CFU/shrimp	2.69×10^5–1.36×10^6
2	1.37×10^6 CFU/shrimp	7.09×10^5–2.64×10^6
3	5.89×10^5 CFU/shrimp	2.90×10^5–1.19×10^6
Mean	8.54×10^5 CFU/shrimp (3.06×10^5/g wet weight)	
Palaemonetes pugio		
1	1.46×10^4 CFU/shrimp	6.22×10^3–3.44×10^4
2	2.16×10^4 CFU/shrimp	2.16×10^4–3.88×10^4
Mean	1.81×10^4tCFU/shrimp (6.08t$\times 10^4$/g wet weight)	

The LD_{50} values are presented as colony forming units (CFU) per shrimp and per gram shrimp wet weight.

A two-way ANOVA test was performed using time, oxygen, and the interaction of time times (*) oxygen as the x values and total hemocyte count (THC) as the response (y) value ($\alpha = 0.05$). An *a posteriori* *t*-test was used to compare means at 48 hours.

RESULTS

LD_{50} Tests

Vibrio parahaemolyticus has dose-response pathogenicity to both *Litopenaeus vannamei* and *Palaemonetes pugio*. Dead or moribund shrimp exhibited signs of Vibriosis, including opaqueness of the abdominal muscle, lethargy, expansion of the chromatophores, and abdominal flexure that peaked at the third abdominal segment (Lightner 1988). LD_{50} values for *L. vannamei* ranged from 5.89×10^5 to 1.37×10^6 CFU per shrimp with an average value of 8.54×10^5 CFU/shrimp (Table 2, $n = 3$). The 95% confidence interval ranged from 2.69×10^5 to 2.64×10^6 CFU/shrimp. Concentrations of bacteria used for the challenge tests remained within these confidence intervals.

LD_{50} values for *P. pugio* were 1.46×10^4 CFU/shrimp and 2.16×10^4 CFU/shrimp, with an average value of 1.81×10^4 CFU/shrimp (Table 2, $n = 2$). The 95% confidence interval ranged from 6.22×10^3 to 3.88×10^4 CFU/shrimp.

Challenge Tests

Control survival

L. vannamei control survival was greater than 77.8, 88.9, and 86.1% in the experiments testing normoxia, normocapnic hypoxia at 45 torr oxygen, and hypercapnic hypoxia at 30 torr oxygen, respectively. *P. pugio* control survival was greater than 92.5, 97.5, and 95% in the experiments testing normoxia, hypercapnic hypoxia at 45 torr oxygen, and hypercapnic hypoxia at 30 torr oxygen, respectively (Fig. 2 and 3). These results show that the levels of hypoxia used were not lethal to either organism.

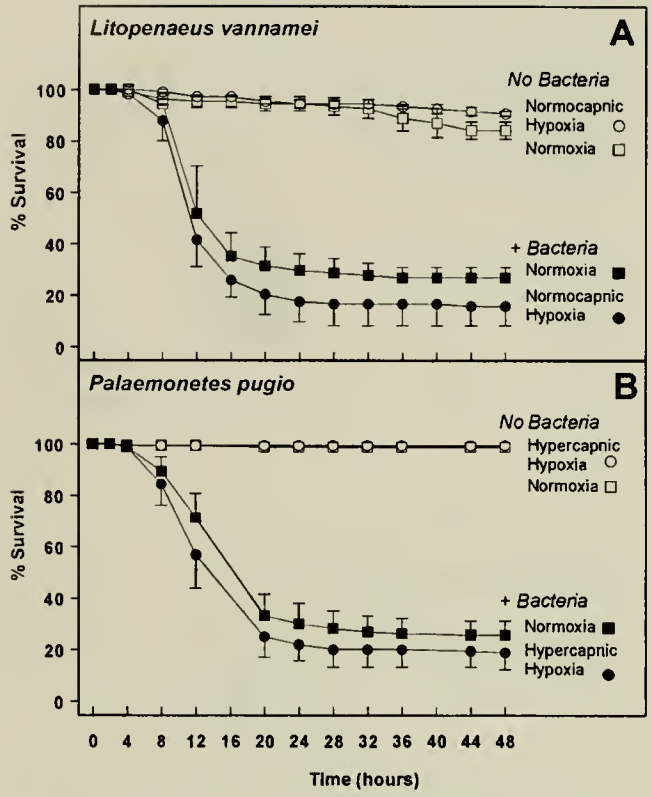

Figure 2. A. *Litopenaeus vannamei* survival following bacterial challenge under normoxia (Po_2 = 150–155 torr, Pco_2 = 0.23 torr, pH 7.6–8.0) and normocapnic hypoxia (Po_2 = 45 torr, Pco_2 = 0.23 torr, pH 7.8–8.1). Shrimp were injected intramuscularly with 50 µL of *Vibrio parahaemolyticus* bacterial suspension (1.8×10^6 CFU/shrimp) or with HEPES buffered 2.5% NaCl for controls. There were 36 shrimp per treatment. Values at each time point are the mean (n = 3 experiments); standard errors are indicated except where the error is small and falls within the width of the datapoint. The effects of oxygen/CO_2 treatment and the interaction of bacteria*oxygen/CO_2 treatment on disease susceptibility were not significant as determined by a MANOVA (P = 0.6478 and P = 0.3594). B. *Palaemonetes pugio* survival following bacterial challenge under normoxia (Po_2 = 150–155 torr, Pco_2 = 0.23 torr, pH 8.0–8.2) and hypercapnic hypoxia (Po_2 = 45 torr, Pco_2 = 15.2 torr, pH 6.9–7.0). Shrimp were injected intramuscularly with 5 µL of *Vibrio parahaemolyticus* bacterial suspension (1.0×10^5 CFU/shrimp) or with HEPES buffered 2.5% NaCl for controls. There were 40 shrimp per treatment. Values at each time point are the mean (n = 4 experiments); standard errors are indicated except where the error is small and falls within the width of the datapoint. The effects of oxygen/CO_2 treatment and the interaction of bacteria*oxygen/CO_2 treatment on disease susceptibility were not significant as determined by a MANOVA (P = 0.7379 and P = 0.7412).

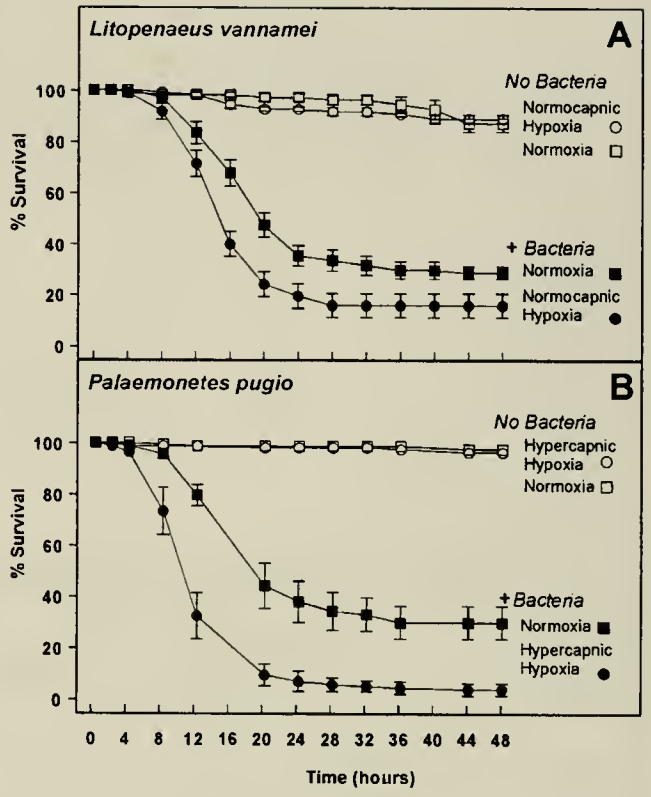

Figure 3. A. *Litopenaeus vannamei* survival following bacterial challenge under normoxia (Po_2 = 150–155 torr, Pco_2 = 0.23 torr, pH 7.6–8.0) and hypercapnic hypoxia (Po_2 = 30 torr, Pco_2 = 15.2 torr, pH 6.8–7.0). Shrimp were injected intramuscularly with 50 µL of *Vibrio parahaemolyticus* bacterial suspension (1.125×10^6 CFU/shrimp) or with HEPES buffered 2.5% NaCl for controls. There were 36 shrimp per treatment. Values at each time point are the mean (n = 3 experiments); standard errors are indicated except where the error is small and falls within the width of the datapoint. The effects of oxygen/CO_2 treatment and the interaction of bacteria*oxygen/CO_2 treatment on survival were significant as determined by a MANOVA (P = 0.0009 and P = 0.0493). Animals held under this level of hypercapnic hypoxia were more susceptible to *Vibrio* challenge than those held under normoxia. B. *Palaemonetes pugio* survival following bacterial challenge under normoxia (Po_2 = 150–155 torr, Pco_2 = 0.23 torr, pH 8.0–8.2) and hypercapnic hypoxia (Po_2 = 30 torr, Pco_2 = 15.2 torr, pH 6.9–7.0). Shrimp were injected intramuscularly with 5 µL of *Vibrio parahaemolyticus* bacterial suspension (9.10×10^4 CFU/shrimp) or with HEPES buffered 2.5% NaCl (controls). There were 40 shrimp per treatment. Values at each time point are the mean (n = 4 experiments); standard errors are indicated except where the error is small and falls within the width of the datapoint. The effects of oxygen/CO_2 treatment and the interaction of bacteria*oxygen/CO_2 treatment on survival were significant as determined by a MANOVA (P = 0.0113 and P = 0.0095). Animals held under this level of hypercapnic hypoxia were more susceptible to *Vibrio* challenge than those held under normoxia.

L. vannamei—normocapnic hypoxia at 45 torr oxygen

These challenge tests examined the effect of moderate hypoxia only (normocapnic hypoxia) without added Co_2 (hypercapnia) on *L. vannamei* survival (Fig. 2A). The effects of oxygen and the interaction of bacteria*oxygen were not significant at Po_2 = 45 torr as determined by a MANOVA (P = 0.6478 and P = 0.3594, respectively). The effect of bacteria alone was significant ($P \leq 0.0001$). Subsequent ANOVA tests revealed that the significance was due to differences in the coefficient and coefficient^2 ($P < 0.0001$ for both). These results indicated that differences in *L. vannamei* survival were attributable to the injection of bacteria over the injection of saline, and not to differences in oxygen levels of the water.

P. pugio—hypercapnic hypoxia at 45 torr oxygen + 15.2 torr (2%) CO_2

These *Vibrio* challenges tested the effect of hypercapnic hypoxia on *P. pugio* survival at a moderate level of hypoxia (45 torr

or 6% O_2) (Fig. 2B). The effects of oxygen/CO_2 treatment and the interaction of bacteria*oxygen/CO_2 treatment were not significant ($P = 0.7379$ and $P = 0.7412$, respectively). The effect of bacteria was significant ($P < 0.0001$) and was attributable to differences in the coefficient and coefficient^2 ($P < 0.0001$ for both, ANOVA). These results show that there was no additional disease susceptibility in *P. pugio* held under this level of hypercapnic hypoxia than those held in normoxic water.

***L. vannamei*—hypercapnic hypoxia at 30 torr oxygen + 15.2 torr (2%) CO_2**

These challenge tests investigated the effects of hypercapnic hypoxia on *L. vannamei* at a more severe level of hypoxia (30 torr or 4% O_2) (Fig. 3A). The effects of oxygen/CO_2 treatment, bacteria, and the interaction of bacteria*oxygen/CO_2 treatment were significant ($P = 0.0009$, $P < 0.0001$ and $P = 0.0493$, respectively) and were attributable to differences in the coefficient and coefficient^2. These results show that *L. vannamei* held under this level of hypercapnic hypoxia experienced a higher rate of mortality from *Vibrio* challenge than shrimp held under normoxic conditions. Average survival at 48 h for animals in normoxia was 28.7 ± 2.4% standard error (SE) versus 15.7 ± 4.6% SE for those in hypercapnic hypoxia.

***P. pugio*—hypercapnic hypoxia at 30 torr oxygen + 15.2 torr (2%) CO_2**

These *Vibrio* challenges tested the effect of hypercapnic hypoxia on *P. pugio* at a more severe level of hypoxia (30 torr or 4% O_2) (Fig. 3B). The effects of oxygen/CO_2 treatment, bacteria, and the interaction of bacteria*oxygen/CO_2 treatment were significant ($P = 0.0113$, $P < 0.0001$, and $P = 0.0095$, respectively) and were attributable to differences in the coefficient and coefficient^2. These results show that *P. pugio* held under this level of hypercapnic hypoxia experienced higher mortality rates from bacterial challenge than animals held under normoxic conditions. Average survival at 48 h for normoxia was 29.4 ± 6.4% SE versus 3.1 ± 2.4% SE for hypercapnic hypoxia.

Total Hemocyte Count

Total hemocyte count significantly decreased in adult *L. vannamei* held under hypercapnic hypoxia when compared to animals held under normoxia over 48 hours. THC/mL was reduced in hypercapnic hypoxia by 60.7, 34.1, 34.3, 40.4 and 16.7% at 4, 8, 16, 24, and 48 hours, respectively, in relation to the normoxia value at the same time point (Fig. 4). A two-way ANOVA indicated that there was a significant effect of oxygen level/CO_2 treatment ($P < 0.0001$) on THC/mL; however, there was no significant effect of time ($P = 0.2907$) or the interaction between time and oxygen/CO_2 treatment ($P = 0.2276$). An *a posteriori* *t*-test used to compare means between oxygen levels at 48 hours revealed that although oxygen level/CO_2 treatment was significant in the two-way ANOVA, THC/mL was not significantly different between the two treatments at 48 hours ($P = 0.3207$, Fig. 4).

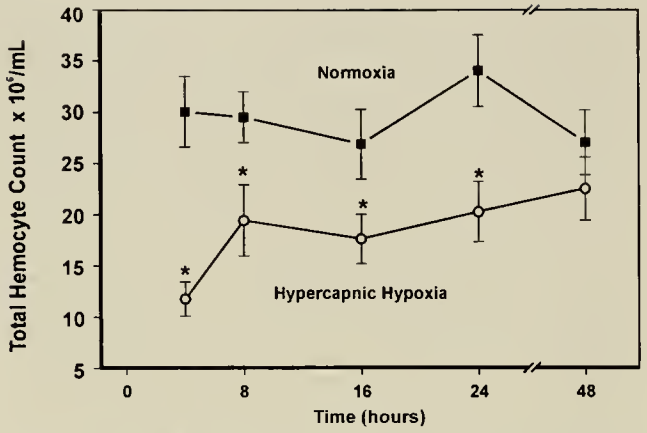

Figure 4. Total hemocyte counts (THC) per mL in *Litopenaeus vannamei* over 48 hours. Shrimp were held under normoxic conditions (closed squares, Po_2 = 150–155 torr, Pco_2 = 0.23 torr, pH 8.0–8.2) or under hypercapnic hypoxic conditions (open circles, Po_2 = 30 torr, Pco_2 = 15.2 torr, pH 6.9–7.1). Individual shrimp were used for each time point and for each test condition (n = 10 shrimp for normoxia and hypercapnic hypoxia at 4, 16, 24, and 48 h, n = 11 shrimp for normoxia and hypercapnic hypoxia at 8 h). A two-way ANOVA indicated that there was a significant effect of oxygen level/CO_2 treatment (P = <0.0001) on THC/mL; however, there was no significant effect of time (P = 0.2907) or the interaction between time and oxygen/CO_2 treatment (P = 0.2276). An *a posteriori* *t*-test revealed that THC/mL was not significantly different between normoxia and hypercapnic hypoxia at 48 hours (P = 0.3207). Values are mean ± standard error (α = 0.05).

DISCUSSION

Estuarine organisms routinely encounter fluctuations in oxygen, carbon dioxide, and pH that may affect their ability to defend against infections. Previous research has linked poor water quality, particularly hypoxia, with increased incidence of infectious disease (Snieszko 1974, Hargis et al. 1989, Landsberg et al. 1998). For example, Landsberg et al. (1998) found that the occurrence of opportunistic protist infections increased in fish subjected to low oxygen conditions. Noga et al. (1994) reported that blue crabs *Callinectes sapidus* collected in areas of the estuary where hypoxia is common, have low serum bacteriostatic activity. This decreased antibacterial activity was correlated with an increase in shell disease. Haley et al. (1967) attributed infections by *Aeromonas liquefaciens* in the threadfin shad *Dorosoma petenense* and the American shad *Alosa sapidissima* to low dissolved oxygen. However, in these field studies, pollutants or other physical factors, such as temperature, may have exacerbated the effects of hypoxia. There have been relatively few studies on the direct effects of hypoxia, hypercapnia, and low pH on disease susceptibility. In the present study, survival following challenge with *Vibrio parahaemolyticus* was depressed in hypercapnic and hypoxic water (Po_2 = 30 torr, Pco_2 = 15.2 torr and pH = 6.8–7.0) in the penaeid shrimp *Litopenaeus vannamei* and in the grass shrimp *Palaemonetes pugio* (Fig. 3A and B). In addition, the THC/mL in *L. vannamei* was reduced under the same conditions (Fig. 4).

The present study used a known pathogenic strain isolated from shrimp with vibriosis. LD_{50} values were reproducible using the same strain and produced consistent mortalities when used in the challenge assays. These are the first reported LD_{50} values for *L. vannamei* and *P. pugio* using *V. parahaemolyticus*.

The 48-h LD_{50} of *V. parahaemolyticus* for *L. vannamei* reported in the present study (Table 2) is similar to the LD_{50} of the same bacterial species for *P. monodon* (3.16×10^5, 95% C.I. 9.60×10^4 to 1.03×10^6 CFU/shrimp). The latter values were calculated from data in Alapide-Tendencia and Dureza (1997) using the trimmed Spearmann–Karber program. Arume (1989) reported LD_{50} values of *Vibrio* isolates to *Litopenaeus stylirostris* ranging from 4.0×10^2 to 3.3×10^4 CFU/g, which is lower than the value

of 3.06×10^5/g wet weight calculated for *L. vannamei* (Table 2). However, the species of *Vibrio* used was not reported. In contrast, *V. parahaemolyticus* had a much higher LD_{50} value for *M. japonicus* juveniles of 4.27×10^7 CFU/shrimp (Vera et al. 1992). This inconsistency in LD_{50} values may be attributable to host specificity and the differences in the size of the animals (Vera et al. 1992, Lee et al. 1996). In addition, virulence of bacteria can vary among strains (Arume 1989, Thune et al. 1993, Wong et al. 1996).

L. vannamei and *P. pugio* were more susceptible to *V. parahaemolyticus* when held under hypercapnic hypoxia at 30 torr oxygen (1.41 mg/L), 15.2 torr (2%) CO_2 and a pH of 6.8 to 7.0 than under normoxia at 150–155 torr oxygen (7.29 mg/L), approximately 0.23 torr CO_2 (0.03%) and a pH of 7.6–8.2 (Figs. 3A and B). This decrease in disease resistance was not attributable to enhanced bacterial growth under these conditions (data not shown). Le Moullac et al. (1999) also found that mortality under hypoxia at 1 mg O_2/L (48%) was significantly greater than control (well-aerated water) mortality (32%) when *L. stylirostris* was challenged with *V. alginolyticus*. However, the levels of CO_2 and the resultant hypoxic pH were not controlled or reported by the investigators. As a result, it is unclear if the animals were subject to hypercapnic hypoxia or to normocapnic hypoxia. In the present study, there was no significant effect on disease susceptibility in *L. vannamei* of normocapnic hypoxia at 45 torr oxygen with less than 1 torr CO_2 or in *P. pugio* under hypercapnic hypoxia at 45 torr oxygen and 15.2 torr CO_2 (Figs. 2A and B).

The level of hypoxia at which disease susceptibility increased (Po_2 = 30 torr) over normoxia in both species may be explained, in part, by the shrimps' critical oxygen tension. The critical oxygen tension for an organism is the oxygen tension below which an organism is unable to maintain its rate of oxygen uptake. Below the critical oxygen tension, organisms may be unable to sustain an internal oxygen level sufficient to defend against infection. Cochran and Burnett (1996) reported a critical Po_2 for *P. pugio* between 30 and 35 torr, which may partly explain differences in susceptibility at 30 torr (1.41 mg O_2/L) and 45 torr oxygen (2.12 mg O_2/L) observed in grass shrimp in this study (Figs. 2B and 3B). On the other hand, Hutcheson et al. (1985) reported a much higher critical Po_2 (approximately 95 torr) for the same species. Nielsen and Hagerman (1998) reported critical Po_2s for *Palaemonetes varians* and *Palaemon adspersus* of 2.4 mg O_2/L (approximately 46 torr) and 2.87 mg O_2/L (approximately 55 torr), respectively, which are both above the highest level of oxygen used in the present experiments. Villarreal et al. (1994) identified a critical Po_2 of 1.3 mg O_2/L (approximately 34 torr) in *L. vannamei*. This value is similar to the value of 1.41 mg O_2/L (30 torr) found to be significant to disease resistance in *L. vannamei* in the present research. In contrast, Rosas et al. (1999) found that juvenile *Litopenaeus setiferus* were oxyregulators down to 4 mg O_2/L (approximately 92 torr), but were oxyconformers between 3 and 2 mg O_2/L (approximately 69 and 46 torr) suggesting that the critical Po_2 lies between those two values. The variability in published critical oxygen pressures may be attributable to many factors including temperature, salinity, activity, molt cycle, size, and experimental technique that can affect the critical Po_2 of a species (Herreid 1980, Dall 1986, Cochran and Burnett 1996).

It is important to note that the oxygen tensions used in the present study were well above the lethal limits reported for these and similar species. Hopkins et al. (1991) reported an oxygen lethal limit of 1 mg O_2/L (approximately 22 torr) for *L. vannamei*. Allan and Maguire (1991) calculated 98-h and 24-h oxygen LC_{50}s for juvenile *P. monodon* of 0.9 mg O_2/L and 0.6 mg O_2/L (approximately 21 and 14 torr), respectively, demonstrating that the duration of the hypoxia also has an effect. Stickle et al. (1989) showed that *F. aztecus* were much more sensitive to low oxygen than *P. pugio*. The 28-day LC_{50} values were 123 torr (5.94 mg O_2/L) for *F. aztecus* and 46 torr (2.22 mg O_2/L) for *P. pugio*. Differences in disease susceptibility between the two species used in the present study (*L. vannamei* and *P. pugio*) could not be compared statistically because of differences in the size of the shrimp and the bacterial challenge dose; however, they exhibited similar responses to the two levels of oxygen tested (Figs. 2 and 3).

As mentioned previously, studies that investigate the effects of hypoxia on estuarine organisms often do not take into account hypercapnia and the low pH that accompanies it (Hutcheson et al. 1985, Seidman and Lawrence 1985, Allan and Maguire 1991, Charmantier et al. 1994, Direkbusarakom and Danayadol 1998, Nielsen and Hagerman 1998, Le Moullac et al. 1999). Nevertheless, these variables may have contributed, in combination or independently, to the decreased disease resistance observed in the present work. Martinez et al. (1998) reported that the lethal dissolved oxygen concentrations for postlarval and juvenile *L. setiferus* are higher under low pH (pH = 6) than under high pH (pH = 8). In addition, McCulloch (1990) found that low pH raised the critical oxygen concentration from 1.54 mg O_2/L at pH 9.0 to 2.08 mg O_2/L at pH 6.5 for *Palaemonetes kadiakensis*. Cochran and Burnett (1996) demonstrated that oxygen uptake was significantly higher at high CO_2 than at low CO_2 in the spot *Leiostomus xanthurus*. Cruz-Neto and Steffensen (1997) reported that hypercapnia increased the critical oxygen concentration from 25 torr to 40–45 torr in the European eel *Anguilla anguilla*. These studies show that hypercapnia can adversely affect hypoxia tolerance.

Total hemocyte count was significantly reduced in *L. vannamei* held under hypercapnic hypoxia (Po_2 = 30 torr, Pco_2 = 15.2 torr, pH 6.8–7.0) when compared to shrimp held under normoxia (Po_2 = 150–155 torr, Pco_2 = 0.23 torr, pH 7.6–8.0) at 4, 8, 16, and 24 h (Fig. 4). Similarly, Le Moullac et al. (1999) found that THC/mL decreased in *L. stylirostris* exposed to hypoxia at 1 mg O_2/L for 24 hours. Alvarez et al. (1989) also noted a reduction in hemocyte concentration in oysters held under two levels of hypoxia (9 and 80 torr oxygen) for 3 days.

Although not measured in the present study, injection of whole bacteria or isolated cell wall components of bacteria and yeast can trigger a decrease in THC/mL in crustaceans (Hauton et al. 1997, Smith et al. 1983, Lorenzon et al. 1999). Using bacterial lipopolysaccharide to suppress circulating THC, Lorenzon et al. (1999) reported threshold lethal limits for THC of 28.9, 32.9, and 15.3% of the initial circulating cells for *P. elegans*, *C. crangon*, and *Squilla mantis*, respectively. These observations suggest that *L. vannamei* and *P. pugio* in the present study that were challenged with bacteria while being held under hypercapnic hypoxia may have experienced a greater decrease in THC than the unchallenged adults held under hypercapnic hypoxia alone. The average total hemocyte count in *L. vannamei* in the present study was reduced to 39.3% of the normoxic average 4 hours after placement in hypercapnic hypoxia (Fig. 4). The combined effects of hypercapnic hypoxia and bacterial injection could reduce cell density in shrimp to a level below the minimum necessary for survival. This is a possible explanation for the increase in mortality in shrimp challenged with bacteria and exposed to hypercapnic hypoxia as compared to animals challenged with bacteria and maintained under normoxia in this study. It is important to note, however, that

dead or moribund animals exhibited signs of vibriosis and that low hemocyte number was not the sole cause of mortality. More likely, the low cell numbers in animals held under hypercapnic hypoxia were insufficient to defend against the bacterial challenge.

The decrease in THC/mL observed in this study may contribute to the increase in mortality rate following bacterial challenge under hypercapnic hypoxia, but many other factors also may play a role. Le Moullac et al. (1999) found that respiratory burst activity, as measured by NBT reduction, decreased in *L. stylirostris* subjected to hypoxia (1 mg O_2/L), but that phenoloxidase (PO) activity increased significantly because of a lower amount of inhibitors regulating the prophenoloxidase system. Direkbusarakom and Danayadol (1998) demonstrated that phagocytosis and bacterial clearance efficiency were reduced in *P. monodon* exposed to 1.8–2.0 mg O_2/L. Although Alvarez et al. (1992) reported that phagocytosis by hemocytes of the eastern oyster *C. virginica* was not affected by hypoxia, Boyd and Burnett (1999) demonstrated that reactive oxygen intermediate (ROI) production by hemocytes was significantly depressed under hypoxia in the same species. Boleza (1999) found that ROI production and bactericidal activity of phagocytes in the mummichog *Fundulus heteroclitis* were suppressed under hypercapnic hypoxia. Comparable studies of cellular and acellular bactericidal factors in *P. vannamei* and *P. pugio* under relevant water quality conditions may clarify the defense mechanisms that are sensitive to dissolved gasses and pH.

The results of the present study show that hypercapnic hypoxia at 30 torr O_2, 15.2 torr CO_2 and a pH range of 6.8 to 7.0 decreases survival following bacterial challenge in both *L. vannamei* and *P. pugio* and decreases total hemocyte count in *L. vannamei* (Fig. 3A and B and 4). This has implications regarding the health of these organisms in both the natural environment and in aquaculture. Diaz and Rosenberg (1995) reported that the occurrence of hypoxia in shallow coastal and estuarine systems is increasing worldwide. Dissolved oxygen monitoring by Summers et al. (1997) in the mid-Atlantic and Gulf of Mexico regions suggests that the extent of hypoxia is often substantially underestimated. Thus, conditions that suppress disease resistance may become more prevalent, which could affect the penaeid shrimp fishery and reduce the density of the ecologically important grass shrimp. In addition, a decrease in THC/mL under hypercapnic hypoxia, as observed in this research (Fig. 4), could result in a decrease in immune function and possibly a reduction of the effectiveness of immunostimulants used in aquaculture to prevent outbreaks of disease (Sung et al. 1991, Sung et al. 1996, Itami et al. 1998, Devaraja et al. 1998, Teunissen et al. 1998). Taken together with the well-recognized importance of water oxygen, dissolved CO_2 and concomitant changes in pH that accompany naturally occurring hypoxia should be carefully monitored and regulated to sustain the wild shrimp fishery and optimize farm production.

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PARALYTIC SHELLFISH TOXINS IN GEODUCK CLAMS (*PANOPE ABRUPTA*): VARIABILITY, ANATOMICAL DISTRIBUTION, AND COMPARISON OF TWO TOXIN DETECTION METHODS

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ABSTRACT The geoduck clam, *Panope abrupta*, is a valuable economic resource in Washington State. Prior to the mid 1970s, the levels of paralytic shellfish poisoning (PSP) toxins in Washington State geoducks were not considered by the Washington State Department of Health (WDOH) to be a risk to public health because the viscera were presumed to be discarded. Recent monitoring information indicates that geoducks accumulate high levels of toxins, primarily in the viscera. The purposes of this study were to determine: (1) the seasonal concentration of paralytic shellfish toxins in geoduck clams at two sites and at two depths within each site; (2) the variability of PSP toxin levels among individual clams within each site; (3) the anatomical distribution of toxins; and (4) the correlation between two methods for estimating PSP toxins. From the summer of 1997 through the winter of 1998, 12–24 geoducks were collected biweekly from a shallow (7 m) and a deep (17 m) location in each of two tracts in Puget Sound, Washington: Quartermaster Harbor (QH) and Agate Pass (AP). Geoducks, dissected into siphon, mantle, and visceral portions, were assayed separately using the mouse bioassay (MBA), while only the visceral portions were assayed using the receptor-binding assay (RBA). Results indicated that toxin variability between individual clams was high in the shallow areas, with coefficients of variation (CVs) ranging from 20–98%, and lower in the deep areas (CV = 18–62%). In QH, only geoducks from the shallow water had toxin levels greater than the regulatory level of 80 μg saxitoxinequivalents (STX eq) · 100 g shellfish tissue^{-1}, while all geoducks from AP contained toxin above the regulatory level, with clams from shallow water considerably more toxic than those from deep water. Anatomically, the highest concentrations of PSP toxins were localized in the viscera of geoducks. There was a significant positive correlation between toxin levels measured by the MBA compared to values obtained using the RBA (r^2 = 0.83). The large differences in toxicity between geoducks sampled at different depths and harvest tracts indicate that careful management plans must be designed in order to ensure public health.

INTRODUCTION

General Background

Toxins that cause paralytic shellfish poisoning (PSP) are accumulated by filter-feeding bivalve mollusks when they ingest toxic dinoflagellates from the genus *Alexandrium*. These algae produce the toxins naturally, and the PSP syndrome results from the human consumption of toxic bivalves. Large-scale problems with PSP stem from the extreme difficulty in predicting the timing and extent of dinoflagellate blooms, in turn making it difficult to monitor toxicity in shellfish efficiently (Boesch et al. 1996, Horner et al. 1997). Difficulties also arise because each species of shellfish is unique in the kinetics of uptake and elimination of toxins. In addition, shellfish toxicities do not always coincide with observed toxic algal blooms (Cembella and Shumway 1993, Bricelj and Shumway 1998).

The geoduck clam, *Panope abrupta*, is a valuable economic resource in Washington State, with revenues ranging from $5–7 million annually (Washington State Department of Natural Resources (WDNR), unpublished data, 1997). Recently, the demand from newly developed large markets both domestically and overseas (e.g., Hong Kong, Japan, and Singapore) has sent the price of geoducks up from $1.50 per pound in the late 1980s to a current average price range of $12–14 per pound. Public demand is for whole, live geoducks; the market for shucked or frozen product is very small. While geoducks are one of many species of bivalves known to filter and accumulate toxic dinoflagellates, few data exist that describe PSP toxins in this organism (Shumway 1990; Bricelj and Shumway 1998). The risk of PSP to consumers is therefore increased, which may lead to devaluation of the geoduck as a food item for human consumption if toxic product reaches the market.

Washington's Geoduck Fishery

Prior to the mid 1970s, PSP toxin levels in Washington State geoducks were not considered by the Washington State Department of Health (WDOH) to be a risk to public health because the geoduck viscera were presumed to be discarded. However, we now know that the viscera are consumed by some members of tribal and immigrant communities, who use them in soup (K. Chew, University of Washington pers. comm., 1996, M. Antee, WDOH pers. comm., 1997). In addition, toxic algal blooms are extending into previously benign areas of central and southern Puget Sound (Nishitani and Chew 1988, F. Cox, WDOH pers. comm., 1997), which is leading to unprecedented high levels of PSP toxicity in geoducks and toxicity that lasts well into the winter months, resulting in thousands of dollars of an unharvestable resource. The recent increased demand for geoduck meat is resulting in new tribal and state commercial tracts being opened in some areas of

central and northern Puget Sound where PSP is known to occur (F. Cox, WDOH pers. comm., 1997).

Little information exists regarding PSP toxicity in geoducks. However, recent monitoring programs indicate considerable inter- and intrapopulation variability (F. Cox pers. comm., 1997).

An understanding of the reasons for toxin variability is crucial in designing a regional monitoring and sampling program. The current method used by the WDOH in monitoring and testing for PSP in the geoduck does not account for individual variability in the clams because composite viscera from three clams are tested for toxicity as one sample. In the absence of variability and anatomical distribution information, it is difficult to assess the effectiveness of the current Washington State geoduck monitoring program in protecting public health. This study describes toxin variability in geoducks in relation to water depth and geographical location, thereby providing basic information that can be integrated into future monitoring efforts by the WDOH.

MATERIALS AND METHODS

Sampling

Quartermaster Harbor

Quartermaster Harbor (QH), located between the southern tips of Vashon and Maury Islands (Fig. 1), is currently a prohibited harvest area due to consistent levels of PSP toxicity > 80 μg of saxitoxin equivalents (STXeq)/100 g of tissue (all toxicities are given in micrograms of STXeq/100 g of shellfish tissue) (Nishitani and Chew 1984) and pollution problems resulting from failing septic systems (Washington Department of Fish and Wildlife 1997). There are two tracts in QH, and tract number 10300 was randomly chosen as the study site (Washington Department of Fish and Wildlife 1997).

A shallow and a deep sampling location within this tract were randomly selected. The depth of the shallow location averaged 7 m, adjusted to mean lower low water, and the deep location averaged 17m (mean lower low water). A diver collected 6–14 geoducks within a circular area approximately 27 m in diameter from both depth locations, at 2-wk intervals from June through October 1997.

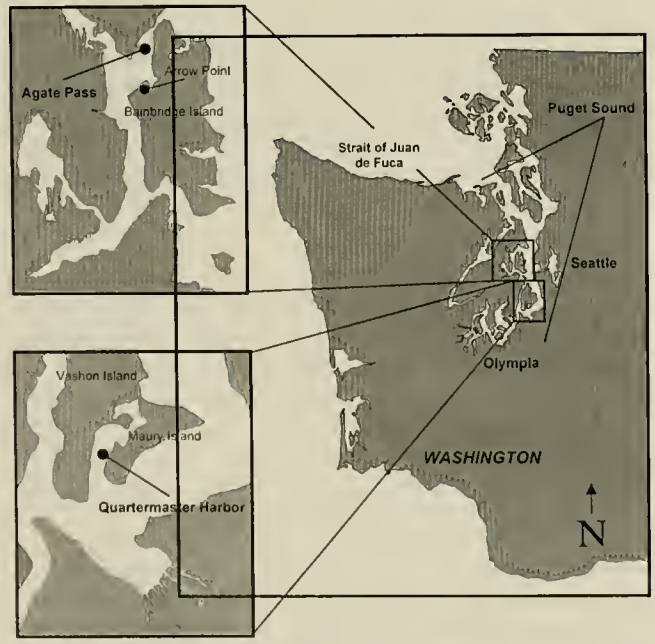

Figure. 1. Map of coastal Washington and Puget Sound showing the study collection sites at AP and QH.

Agate Pass

Tract number 0700 in Agate Pass (AP), located north of Arrow Point on the west side of Bainbridge Island (Fig. 1), is currently a WDOH-approved harvest tract (Washington Department of Fish and Wildlife 1997). A shallow and a deep sampling location were randomly selected in the same manner as in QH. Divers collected geoducks at 2-wk intervals from August 1997 through January 1998. All of the geoducks from deep water consistently came from the same sampling location. In the shallow zone, however, the lack of sufficient numbers of geoducks necessitated a constant lateral shift in collection sites, but all of the shallow sites were within an approximately 300-m section along the shoreline.

Laboratory Determinations

Geoducks were dissected, and toxicities of the siphon, mantle, and visceral portions of individual geoducks were determined by mouse bioassay (MBA) (Association of Official Analytical Chemists 1965). All of the visceral tissue, except the gills, was combined and tested. The gills were saved for future testing, time and funding permitting. Additionally, the visceral portions were tested using the receptor-binding assay (RBA) (Davio and Fontelo 1983, Doucette et al. 1997, Trainer and Poli, 2000). In this assay, nerve terminal membrane from the rat brain, containing sodium channel receptors (STX binding sites) is used to test for the presence of STXeq in a sample. Toxin in the sample displaces radioactively labeled STX from its specific receptor sites, thereby reducing the level of radioactivity in the shellfish sample. Geoduck samples analyzed using this method had toxin levels ranging from 40–1,800 μg (determined by MBA). No samples below the detection limit of the MBA were used.

RESULTS

Anatomical Distribution

The actual toxin levels (given in micrograms of STXeq per 100 g of shellfish tissue) in each of the dissected tissues (siphon, mantle, and viscera) from all clams collected from QH and AP (n = 361), are shown in Figure 2. In QH samples, detectable levels of toxins were found in the mantle portion of three individual clams, but the values were well below the fishery closure level (80 μg) at 46, 47, and 51 μg (Fig. 2B). In AP samples, detectable levels of toxin were found in the mantle portion of seven individual clams and in the siphon portion of nine individual clams (Fig. 2B), however, the values were again well below the fishery closure level. At no time during the study period did the siphon portion from any geoduck show detectable levels of toxicity. All toxicities above the fishery closure level were in the visceral portion only.

Quartermaster Harbor

Shallow Water

Toxin levels above the fishery closure level were detected on all eight sampling dates from June through October, except July 27

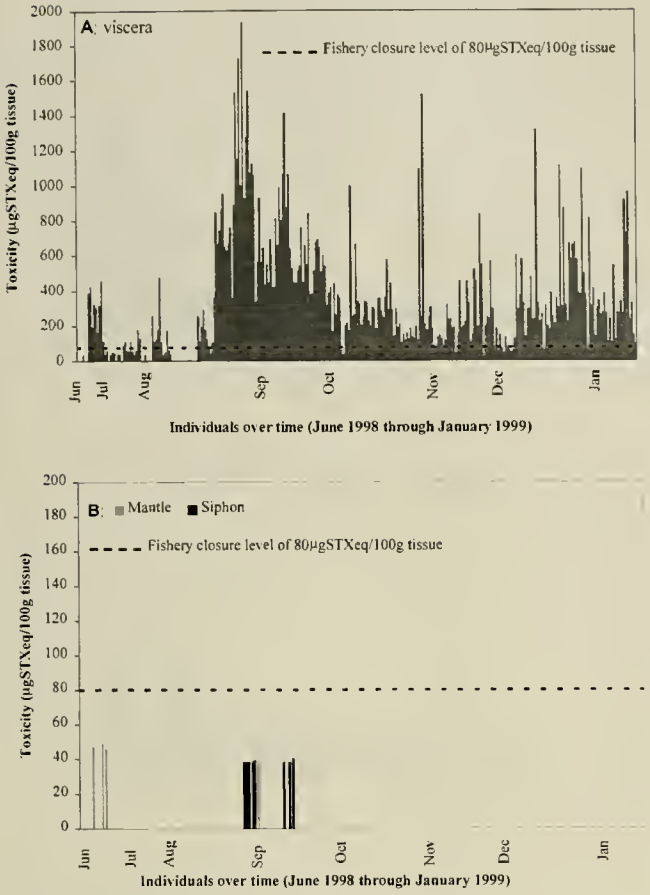

Figure. 2. Toxicity levels in each of the dissected tissues (A = viscera; B = mantle and siphon). Each bar represents an individual geoduck. All geoducks collected from QH and AP during the study are included (n = 361) and are shown in chronological order of collection. The mantle portions of 10 geoducks and the siphon portion of 9 geoducks had detectable levels of toxin but were still below the fishery closure level.

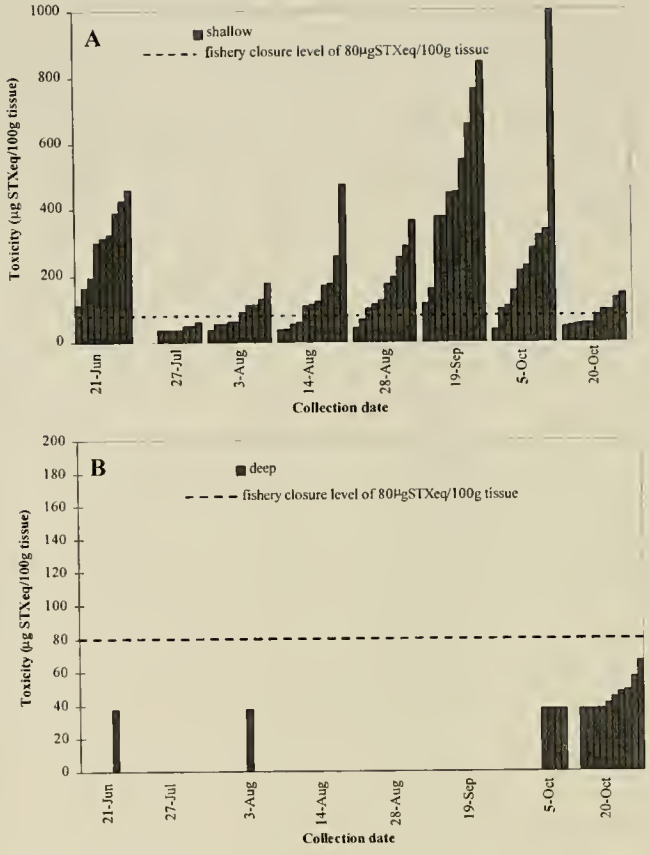

Figure. 3. Toxicity of the viscera in geoducks from the QH collection areas taken from June through October 1997. Each bar represents an individual geoduck. The dashed line indicates the regulatory closure level of 80 μg STXeq/100 g of shellfish tissue. A = shallow; B = deep.

(Fig. 3A). When toxicity was above closure levels, there was a large variation in toxin levels among individual clams. On July 27, variability was low and toxicity levels ranged from 0–61 μg. The largest variation occurred on October 5, with toxin levels ranging from 38–998μg (Table 1).

Deep Water

In QH deep water, toxicity was consistently below the closure level, and values were considerably lower than those observed in the shallow location (Fig. 3B). Toxin levels ranged from nondetectable to 38 μg on all collection dates except October 20, when toxin levels ranged from 0–67 μg. Variability between individuals was low on all sampling dates.

AP

Shallow Water

Toxicities were consistently above the fishery closure level on all 12 collection dates from August through January with the exception of three individual clams, one each on November 12 and 25, and January 6 (Fig. 4A). There was a large variation in toxin levels among individuals on all sampling dates.

Deep Water

Toxicities were consistently above the fishery closure level of 80 μg on most of the 11 collection dates, except December 10 when 50% were below and 50% were above the closure level of 80 μg (Fig. 4B). There was a large variation in toxin levels among individuals on all sampling dates. The largest variation occurred on January 20 with toxicities ranging from 107–545 μg.

Comparison of PSP Detection Methods

Correlations between the MBA and the RBA methods are shown in Figure 5. Figure 5A illustrates the relationship between all samples tested, which ranged from 60–1,700 μg (by MBA). A comparison of the two methods showed a significant positive correlation (r^2 = 0.83). Figure 5B illustrates the relationship between samples with toxicities < 85 μg (by MBA) and demonstrated a significant positive correlation between the two methods (r^2 = 0.55).

DISCUSSION

Variability

The high degree of toxin variability observed among individual geoducks (Figs. 3, 4) is not surprising and has been seen in many other shellfish species. For example, Atlantic surfclams (*Spisula solidissima*) taken off the coast of Maine showed an average coefficient of variation (CV) of 48.6%, and ocean quahogs (*Arctica islandica*) showed a mean CV of 56% (White et al. 1993). Soft-

TABLE 1.

Summary of the variation in levels of PSP toxins among individual geoducks collected from QH and AP during each 1-d collection period, with clams separated by depth.

Area	Sampling Date	*n*	Geoducks with >80 µg of Toxin	Range[a]	Mean ± SD[a]	Pooled SD	CV (%)	Mean CV (%)
QH shallow	June 21	9	9	113–460	298.8 ± 119		40	
	July 27	10[c]	0	0–61	53 ± 7		13	
	August 3	11	6	39–179	95 ± 43		44	
	August 14	11	7	38–475	158 ± 130		82	
	August 28	10	8	44–365	173 ± 103		60	
	September 19	10	10	116–845	474 ± 237		50	
	October 5	10	9	38–998	305 ± 274		90	
	October 20	10	5	46–146	81 ± 36	153	44	53
QH deep	June 21	6	0	0–38	N/A[b]		N/A[b]	
	August 3	6	0	0–38	N/A[b]		N/A[b]	
	August 14	14	0	0	N/A[b]		N/A[b]	
	October 5	10	0	0–38	N/A[b]		N/A[b]	
	October 20	11[d]	0	0–67	51 ± 9	N/A	18	N/A
AP shallow	August 19	12	12	892–1,937	1,272 ± 335		27	
	September 2	10	10	530–1,413	885 ± 258		29	
	September 17	9	9	290–692	476 ± 160		34	
	October 7	9	9	203–666	334 ± 140		42	
	October 14	10	10	102–577	289 ± 143		49	
	October 28	5	5	172–1,521	649 ± 621		96	
	November 12	6	5	49–318	202 ± 88		44	
	November 25	10	9	38–835	398 ± 219		55	
	December 9	14	14	138–1,314	408 ± 292		72	
	December 23	13	13	224–1,113	606 ± 288		48	
	January 6	11	10	61–813	346 ± 195		57	
	January 20	10	10	98–966	431 ± 318	271	74	52
AP deep	August 19	10	10	359–958	717 ± 164		23	
	September 2	11	11	342–930	546 ± 166		30	
	September 17	12	12	212–544	409 ± 107		26	
	October 7	11	9	38–441	247 ± 115		46	
	October 14	11	11	183–357	271 ± 61		23	
	October 28	10	9	116–195	144 ± 29		20	
	November 12	10	10	81–304	151 ± 74		49	
	November 25	10	10	106–521	278 ± 150		54	
	December 9	12	6	47–183	92 ± 44		48	
	December 23	10	9	65–386	252 ± 94		37	
	January 20	10	10	107–545	233 ± 144	115	62	38

[a] Values given as micrograms of STXeq/100 g of shellfish tissue.

[b] N/A = not applicable. These values were below detection level and could not be determined.

[c] Only three geoducks had toxicities >38 µg. These values were used to calculate mean, SD, and CV.

[d] Only six geoducks had toxicities >38 µg. These values were used to calculate mean, SD, and CV.

shell clams (*Mya arenaria*) from the Bay of Fundy showed an average CV of 49% (Medcof et al. 1947). Prior to the present study, the only variability information available for geoducks was from an unpublished study in Alaska, where the mean CV for 10 sets of geoducks was 41% (Ketchikan Public Health Laboratories, unpublished data, 1981).

Some variability in PSP toxin levels among individual geoducks can be accounted for by the variability (± 20%) in the MBA test (McFarren 1962). The mean CVs for each set of geoducks (defined by collection area and depth) were close to or greater than twice that in the MBA (38%, 52%, and 53%). However, within sets of geoducks, the CV reached 96% (Table 1), indicating that there was considerable variability between individual geoducks that was not due to an error in the MBA.

Many factors have been suggested to account for variations between individual shellfish, including differences in feeding rates, availability of food due to vertical and horizontal depth gradients, reproductive condition, individual sensitivity to PSP toxins, and variation in body mass (Prakash and Medcof 1962, Nishitani and Chew 1984, Bricelj et al. 1991, Bricelj and Laby 1996, Mackenzie et al. 1996). Much of the variation between individual geoducks within one depth may be attributable to differences in feeding rates (D. Williams, WDNR pers. comm., 1997). At any given time, geoducks are expected to have a 70% "show factor," meaning that only 70% of the population will have their siphons protruding out of the sand but will not necessarily be feeding. This show factor varies with the time of year and could be attributed to changes in water temperature or localized disturbances (e.g., the presence of divers, crabs, siphon-nipping fish, or marine mammals), causing the geoducks to retract their siphons.

The availability of food, often directly related to the behavior of algal cells, is very likely to be the reason for the high degree of

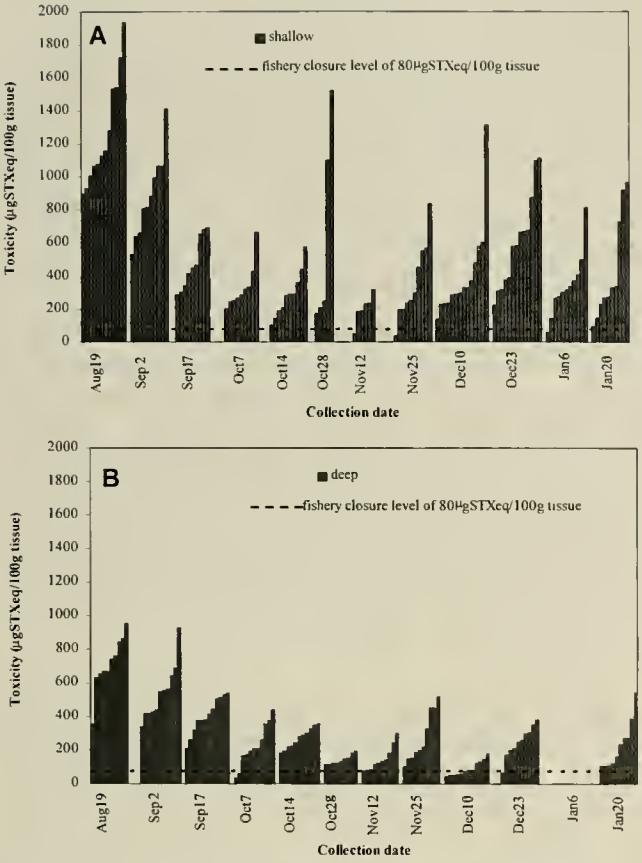

Figure. 4. Toxicity of the viscera in geoducks from the AP collection areas taken from August 1997 through January 1998. Each bar represents an individual geoduck. The dashed line indicates the regulatory closure level of 80 μg STXeq/100 g of shellfish tissue. A = shallow; B = deep.

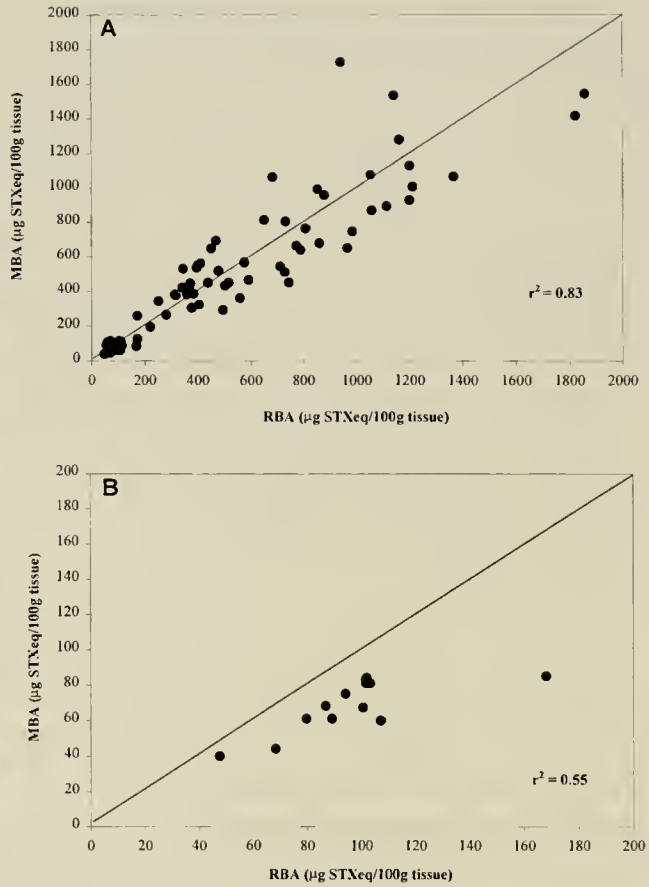

Figure. 5. Correlation between the MBA and the RBA. A = all samples analyzed (n = 73); B = samples with toxin levels between 32 and 85 μg (by MBA, n = 12). The diagonal line represents perfect correlation (r^2 = 1). There was a significant correlation between the two methods: r^2 = 0.83 for all samples and r^2 = 0.55 for samples < 85 μg.

variability between depths (shallow and deep). The toxic *Alexandrium* cells have been found to undergo diel vertical migrations, reaching a maximum depth of 8 m (Nishitani and Chew 1984). The depth of the dinoflagellates also depends on currents and winds, which mix them deeper into the water column. This may explain why the geoducks from the deep water of QH, a shallow, quiet bay without strong currents or vertical mixing, were never over the toxicity closure level, while the ones from the shallow water exhibited high toxin levels (Fig. 3). It is likely that the geoducks from the shallow water were exposed to the toxic dinoflagellates more frequently than the ones from the deep water, thus increasing their overall toxicity. Because AP experiences mixing due to strong and variable currents, with speeds ranging from 0.3–6.6 knots (U.S. Department of Commerce 1973), cells are mixed to greater depth within the water column, making them available for uptake by the geoducks in deeper areas. However, it is likely that the geoducks from the shallow water were exposed to toxic cells more frequently, accounting for their higher overall toxicity.

The difference between collection depths has some implications for the geoduck industry. Currently, harvesting for the market and collection for PSP monitoring occurs primarily in the shallower depths of a harvest tract (D. Winfrey, Puyallup Tribe pers. comm., 1997, D. Williams, WDNR pers. comm., 1998). Geoducks are easier to find, and the divers can collect more clams in a shorter amount of time. In order to accommodate the toxicity differences, harvests could be limited to certain depths during periods of high toxicity (typically in the summer through early winter months). For example, as toxicity levels increase, harvest depths could also be required to increase.

The large difference in overall toxicities between AP and QH is difficult to explain (Figs. 3, 4). It is not unreasonable to suspect that QH would have higher toxicities since it is a "breeding bay" for *A. catenella* (Nishitani and Chew 1984). Breeding bays are defined as certain shallow, protected bays in which strong thermal stratification occurs relatively frequently. In these bays, dense populations of *A. catenella* can develop and become available to the shellfish. However, the AP study area had toxicity levels up to five times (Table 1) those seen in the QH study area, even though AP is an area of strong currents and very little thermal stratification. One explanation for the higher toxicities in AP is the possibility of dinoflagellate cysts in this area, which can be more toxic than motile cells (Dale et al. 1978). Since *A. catenella* forms dormant cysts, it is possible that the geoduck harvesters or the strong currents in AP are stirring up the toxic dormant cysts in the sediments, making them available for uptake by the geoduck and, thereby, accounting for the higher overall toxicities in that area. In addition, QH is a closed harvest area with no diver activity and slow currents, further supporting the explanation for lower overall toxicities.

An additional explanation for higher toxicities in AP could be that motile toxic cells are potentially being exported from a nearby breeding bay. Puget Sound, a fjord with a long, deep main channel, has numerous relatively shallow and often poorly flushed bays, where blooms of *A. catenella* could potentially originate (Horner et al. 1997). The toxic cells, if exported horizontally during periods of reduced turbulence, could cause toxicity levels in shellfish in a nearby area to be several times greater than in the breeding bay itself (Nishitani and Chew 1984).

Individual shellfish within the same sampling population are known to exhibit differential sensitivities to PSP toxins (Bricelj and Laby 1996). This has not been studied in geoducks. Variations in body mass also may have effects on individual toxin accumulation rates. Smaller individual clams can reach equal or higher toxicities than larger individuals collected from the same location (Medcof et al. 1947, Aalvik and Framstad 1981). In this study, there was no relationship between geoduck weight and toxicity level on any date or in any collection area.

Anatomical Distribution

In this study, all PSP toxin levels above closure level were concentrated in the visceral ball in geoducks from all collection sites (Fig. 2). Only the siphon and mantle portions contained an amount of toxin that was below regulatory levels, and, therefore, these were the only portions that would be considered safe to consume during periods of PSP intoxication. This raises the important question of: "How do we protect the public health from the dangers of consuming toxic geoduck viscera?"

The current program used by the WDOH in monitoring for PSP in geoducks could be modified to better protect public health and will be discussed in the next section. Second, geoducks could be shucked and eviscerated prior to being sent to the market. However, 80% of the current market, both domestic and overseas, is for whole, live geoduck, where consumers often pay $12 or more per pound. There is not a large demand for processed geoduck meat (J. Lo, Evergreen International Food Stuff, pers. comm., 1999 L. Elliott, E.C. Phillips and Son, pers. comm., 1999). Typical prices for shucked body meat range from $3–5 per pound, and for neck meat, from $12–24 per pound. These prices depend mainly on the economy and on the availability of whole, live product. In addition, the volume is so small that even the high prices for neck meat do not make up for the overall value of live product. A third solution to the question of how to protect public health is to increase public awareness and education on the dangers of consuming toxic geoduck viscera.

PSP Monitoring

The large difference in toxicity levels between depths and between tracts has implications for the industry and the WDOH. Each harvest area will have to be treated separately when determining sample size and PSP monitoring effort. Perhaps a larger number of samples could be taken in the shallow areas, since most harvest activity occurs in those zones. Because sites vary widely in wind patterns, bathymetry, tidal currents, and turbulence, the extent to which toxicity differences will actually occur may also be expected to vary considerably and can best be tested on a site-by-site basis. It must be noted that the information gained from this study is only applicable to the specific study sites. However, generalizations, such as high variability between individual geoducks, can be made to other populations.

Assay Comparison

In this study, the RBA overestimated MBA results by an average of 22.8%. Doucette et al. (1997) found that the RBA agreed very closely with MBA results from one laboratory but tended to overestimate those originating from a second source. The reasons for overestimation are unclear. Differences are expected, given that the RBA is performed on a static system and the MBA is performed on a dynamic system (live mice). Resulting toxicities can be affected by metabolic changes in the mice. Other work has shown that the MBA is known to underestimate actual toxicity by as much as 60% at lower toxicity levels (McFarren 1957, Park et al. 1986). Therefore, the lower levels of toxicity obtained by MBA in this study could have been underestimated by as much as 60%, accounting for most of the overestimation by the RBA. Variability at low toxicity levels in the MBA is affected by many factors, including salt content during sample preparation, pH, and storage (McFarren 1957, Park et al. 1986). Last, after initial sample preparation, some degradation of the low-toxicity compounds B1 and B2 (N-sulfocarbamoyl toxins) to the nonsulfated carbamate toxins, STX and neosaxitoxin, could have occurred, resulting in increased toxicity by the RBA (Cembella et al. 1993).

The overestimation of toxicity levels by the RBA has implications for the industry and the WDOH if this were chosen as the approved method of toxin detection. At very low levels of toxicity (near the regulatory level of 80 μg), the geoduck fishery would be closed to harvest more often. However, the RBA could prove to be a useful tool in prescreening shellfish for PSP toxins. It also may have applications as a diagnostic tool in suspected cases of STX poisoning in humans and marine animals. Overall, the two methods were in very good agreement, as confirmed by a significant correlation coefficient (r^2 = 0.83 for all samples, r^2 = 0.55 for samples < 85 μg). The assay warrants consideration as a rapid, reliable, and cost-effective alternative to the MBA.

CONCLUSIONS

1. Geoducks collected at shallow depths in both tracts were more variable in levels of toxicity and were more toxic than geoducks from the deeper waters.
2. Toxicity levels in the shallow AP area were about two times those in the shallow QH area. Toxicities in the deep AP area were about five times those in the deep QH area. In the deep AP area, toxicity levels were almost always well above the closure level, while those in the deep QH area were always below closure level.
3. Results indicate substantial variability in toxicity levels among individual geoducks within a small population. It appears that the overall variability among geoducks in both shallow areas can be generally characterized as having a CV of about 53%, and in the AP deep area having a CV of 38%. In the QH deep area, the CV could not be measured because of an insufficient numbers of geoducks with detectable levels of toxicity.
4. All toxin levels recorded above the regulatory closure level (80 μg STXeq/100 g of tissue) were in the viscera only.
5. At low levels of toxicity (< 85 μg STXeq/100 g of tissue), the RBA overestimated the MBA. However, most of the overestimation can be accounted for by the inherent variability in the MBA and its tendency to underestimate low levels of toxicity by as much as 60%. Overall, the two

methods had a high degree of correlation (r^2 = 0.83 for all samples, r^2 = 0.55 for samples < 85 μg STXeq/100 g of tissue).

The results of this research have implications for the geoduck industry and public health agencies. The following recommendations can be implemented to improve geoduck sampling and analysis.

1. Due to the toxicity differences in harvest depth, the collection of geoducks during the PSP season could be limited to the deeper areas of a harvest tract to avoid fishery closures.
2. Farmers interested in culturing subtidal geoducks should consider doing so in deeper areas to avoid the high toxicities found in the shallow areas.
3. From a risk-management standpoint, a larger number of samples collected from shallow areas would have to be analyzed to reduce the risk of PSP intoxication in consumers.
4. The toxicity difference between tracts implies that the physical aspects of each tract may have to be considered when sampling and monitoring for PSP in geoducks.
5. Geoducks should be tested for PSP on an individual basis rather than as a composite of three samples, to account for the high degree of individual variability seen in this study.
6. The viscera could be immediately removed and discarded prior to consumption of the siphon and mantle portions, which have been shown to be safe to consume even during times when viscera are highly toxic.

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VIABILITY OF THE TOXIC DINOFLAGELLATE *PROROCENTRUM LIMA* FOLLOWING INGESTION AND GUT PASSAGE IN THE BAY SCALLOP *ARGOPECTEN IRRADIANS*

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ABSTRACT Bay scallops *Argopecten irradians* were fed cells of the epibenthic dinoflagellate *Prorocentrum lima*, a known producer of diarrhetic shellfish poisoning (DSP) toxins, in controlled microcosms in the laboratory. Examination of scallop fecal ribbons revealed that ingested *P. lima* cells were capable of survival and cell division following passage through the scallop gut. This implies that viable *P. lima* cells released via fecal deposition from transferred stock may be capable of long-term survival and growth. The ability of this dinoflagellate to adapt and survive in a wide variety of benthic environments coupled with circumstantial evidence of its involvement in toxic events, suggests that release of viable cells poses a risk of increasing the geographical range of this species, with negative consequences for the shellfish industry.

KEY WORDS: *Argopecten*, bay scallop, *Prorocentrum*, dinoflagellate, DSP toxins

INTRODUCTION

Contamination of bivalve mollusks by diarrhetic shellfish poisoning (DSP) toxins poses an economic threat for shellfish harvesters in many parts of the world (Shumway 1990). Dinoflagellates recognized as producers of DSP toxins include several planktonic *Dinophysis* species (Reguera et al. 1993, Yasumoto 1990) and a few benthic/epibenthic species of *Prorocentrum* (McLachlan et al. 1994). Although species of *Dinophysis* are implicated most frequently as the causative organisms of DSP events, there is growing evidence that *Prorocentrum lima* (Ehrenberg) Dodge is the cause of DSP toxicity at aquaculture sites in eastern Nova Scotia (Jackson et al. 1993, Lawrence et al. 1998).

The possibility of toxigenic dinoflagellate cells surviving passage through the digestive tracts of shellfish is an area of special concern for growers. After contamination by toxic dinoflagellates, shellfish stocks may be transferred to "clean" waters to depurate (Haamer et al. 1990, Silvert and Cembella 1995). If live dinoflagellate cells are released into pristine waters via fecal deposition from newly transferred stock, they may divide and populate the transfer site. Furthermore, even in the absence of measurable toxicity, there is a possibility that viable cells could be inadvertently transferred to new sites via the expanding trade in cultured shellfish, especially when the apparent increase in the frequency and variety of harmful algal blooms is considered (Hallegraeff 1993). Studies demonstrating the survival of vegetative cells and pellicular cysts of the dinoflagellate *Alexandrium tamarense* (Scarratt et al. 1993), *A. fundyense* (Bricelj et al. 1993), and *A. minutum* (Laabir and Gentien 1999), producers of paralytic shellfish poisoning (PSP) toxins, in mussel (*Mytilus edulis*) and oyster (*Crassostrea gigas*) feces have warned that such a process could serve as a potential seed source for subsequent dinoflagellate blooms.

Recent studies have demonstrated that DSP toxins are accumulated in tissues of the mussel *M. edulis* (Pillet et al. 1995) and the bay scallop *Argopecten irradians* (Bauder et al. 1996) when toxic *P. lima* cells are ingested. However, the fate of undigested cells is also of interest, because no studies have examined whether dinoflagellates known to produce DSP toxins can survive gut passage in bivalves. The objective of the present study was to investigate the likelihood of survival of *P. lima* cells following ingestion by *A. irradians*.

METHODS

Bay scallops *Argopecten irradians* (mean shell height = 38 ± 4 mm), were exposed to toxigenic *Prorocentrum lima* (strain Pa) at a constant density of 10^5 cells L^{-1} for 13 d in an 80-L aquarium at 17 °C (Bauder 1997). Cells were kept in suspension using two recirculating pumps mounted on the aquarium. Scallops were subsequently transferred to another aquarium and depurated for 1 week on a nontoxic diet of the diatom *Thalassiosira weissflogii* (ACTIN, CCMP #1336). During the depuration period, the entire volume of the aquarium was replaced each day with 1 μm-filtered seawater to reduce the possibility of scallops re-ingesting fecal ribbon contents.

Fecal ribbons produced by scallops after 12 days of exposure to *P. lima* cells were gently removed from the aquarium by a Pasteur pipette and rinsed by allowing the feces to settle in 20 mL scintillation vials containing 1-μm filtered seawater. Feces were then transferred to another vial containing filtered seawater and kept on ice.

Viability of *P. lima* cells within fecal ribbons was determined by inoculating triplicate samples of intact or disrupted (vortex-mixed for 30s) fecal ribbons in flasks containing 200 mL of K-medium (Keller et al. 1985). A 5 mL sample of *P. lima* stock culture was also inoculated in triplicate as a control for comparison of cell division rates. Cultures were grown on a 14:10 L:D photocycle at a photon flux density of 90 μmol m^{-2} s^{-1} at 17 °C. Cell concentrations in each of the flasks were determined every 7days over a 4-week period by enumerating 5 mL subsamples in a 0.1 mL Palmer–Maloney chamber under phase-contrast microscopy (100×). Division rates were calculated according to the formula of Guillard (1973).

Fecal ribbons produced by scallops during exposure to *P. lima* cells and during the depuration period were photographed at up to 1000× magnification under Nomarsky interference microscopy. *Prorocentrum lima* cells in the feces were examined for such obvious characteristics as thecal integrity and cell motility.

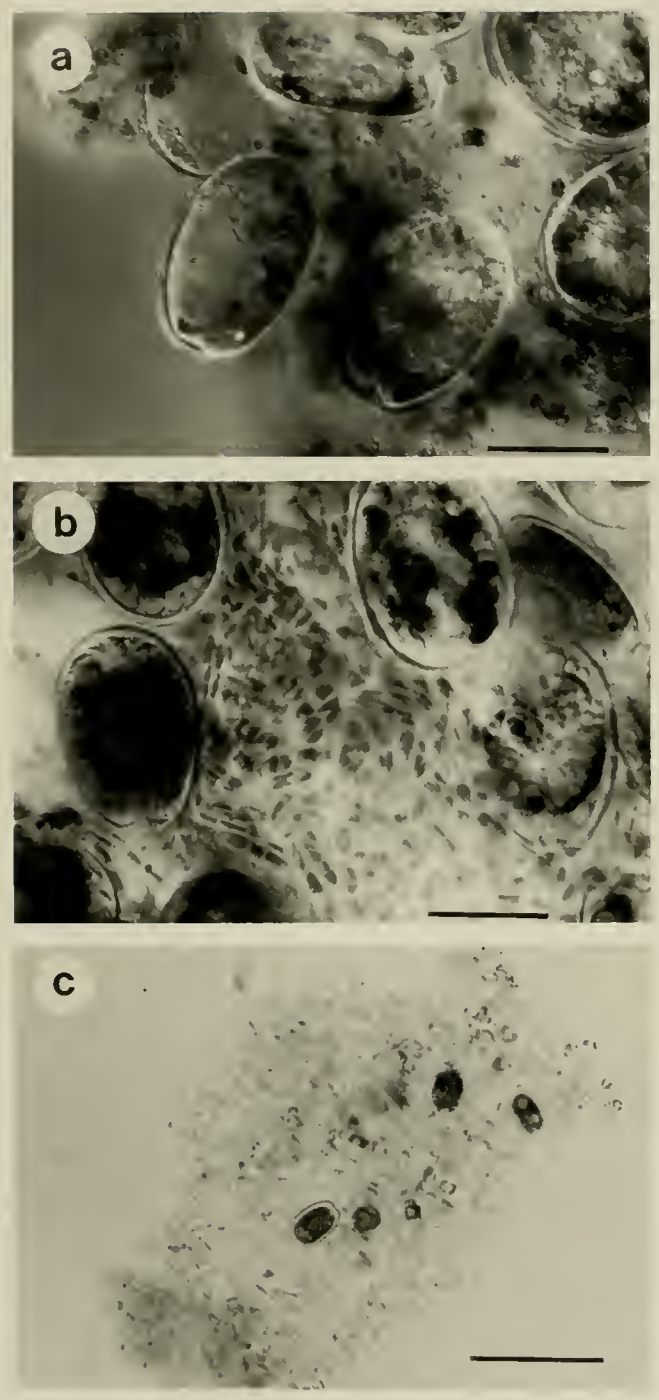

Figure 1. Nomarsky interference photomicrographs of fecal ribbons produced by *Argopecten irradians* after (a) 2 days and (b) 12 days exposure to *Prorocentrum lima*, and after (c) 3 days of depuration on a diet of *Thalassioisra weissflogii*. Scale bars = 20 µm (a, b) and 100 µm (c).

RESULTS

Microscopic examination of fecal samples revealed that intact and motile *Prorocentrum lima* cells were abundant in scallop fecal ribbons throughout the exposure period. Cells were frequently swimming by flagellae within the fecal ribbons, as well as along the exterior margins of the ribbons, suggesting that these cells had recently migrated from the feces into the surrounding medium. Evidence that some *P. lima* cells were digested during passage through the scallop gut was provided by the presence of *P. lima* thecal fragments and free starch granules within the feces. A greater proportion of digested *P. lima* cells within fecal ribbons was observed at the beginning of the exposure period than during the latter portion (Fig. 1).

Live *P. lima* cells were observed by microscopy in fecal ribbons produced by scallops until the fourth day of depuration (Fig 1c). Pigments and thecal fragments derived from digested *Thalassiosira weissflogii* cells were abundant in fecal ribbons; however, unlike *P. lima* cells, very few of these diatom cells were intact.

Mean division rates (k) calculated over exponential growth phase of *P. lima* cells contained in fecal ribbons were 0.26 d^{-1} (SD = 0.03) and 0.21 d^{-1} (SD = 0.04) for disrupted and undisrupted fecal ribbons, respectively, indicating that the cells were viable and able to divide at rates comparable (ANOVA, $P > 0.05$) to those of *P. lima* cultures (k = 0.24 d^{-1}, SD = 0.01) when inoculated into growth medium (Fig. 2). Similar division rates for cells inoculated from intact and dispersed fecal ribbons show that *P. lima* cells were capable of freeing themselves from the fecal ribbons.

DISCUSSION

The present study is the first to demonstrate the survival of DSP-toxigenic algal cells in bivalve fecal ribbons. Although Mackenzie (1998) found large numbers of intact thecae of another DSP toxin producer, *Dinophysis acuta*, in fecal pellets deposited by the mussel *Perna canaliculus*, viable cells were not observed. It was further noted that mussel stomach fluids rapidly lysed *D. acuta* cells.

In a study demonstrating that PSP toxin-producing *Alexandrium* cells could survive and reproduce following passage

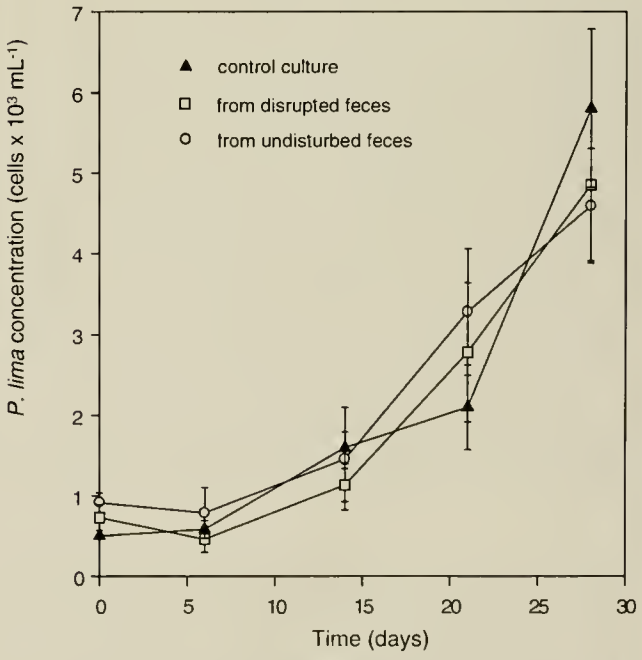

Figure 2. Growth of *Prorocentrum lima* cells in K-medium. Inoculated from either *P. lima* culture or fecal ribbons of *Argopecten irradians* following ingestion of *P. lima* cells (undisturbed and disrupted fecal ribbons). Error bars: ± 1 standard deviation, $n = 3$.

through the gut of *Mytilus edulis*. Scarratt et al. (1993) reported that after 3.9 h of depuration, almost all of the fecal ribbons were free of *Alexandrium* cells. These authors suggested that 12 h of purging should be sufficient to rid mussels of toxic cells before transferring stock to new waters. Passage of *Prorocentrum lima* cells in bay scallop guts occurred over a much longer time scale, as demonstrated by the presence of live cells in fecal ribbons even after 3 days of depuration, during which the scallops were continually ingesting *Thalassiosira weissflogii* cells.

Bomber et al. (1988) reported that live *P. lima* cells have been found attached to the viscera of tropical fish and argued that the ability of these cells to survive in fish viscera for extended periods represents an important system of dispersal for this species. Similarly, the slow passage of *P. lima* cells in scallop guts represents a potential mechanism of cell dispersal to new aquaculture and wild harvest sites via shellfish stock transfer.

Although *P. lima* cells released in scallop feces have been shown by this study to be viable in culture, the analogous situation at a shellfish aquaculture site must be addressed. In the absence of a strong vertical mixing component, bivalve fecal ribbons containing *P. lima* cells would rapidly sink out of the water column to the benthic environment below the site. For many species of photosynthetic microalgae, this environment would be unfavorable for survival, because it is characterized by low irradiance and temperatures as well as enhanced levels of ammonium and organic detritus derived from shellfish waste products. However, observations consistently indicate that *P. lima* is a very robust species, well suited to a wide variety of benthic environments.

Bomber et al. (1985) observed survival of *P. lima* among benthic detritus in unaltered seawater for up to 6 months. Similarly, McLachlan et al. (1994) reported that *P. lima* cultures can be kept in the same culture medium for over 6 months and that the cells are capable of long-term survival at temperatures of 0 °C. The cellulose theca of *P. lima* is extremely resistant to physical disruption; overwintering stages are primarily in the form of vegetative cells, rather than cysts. This demonstrates the potential for this species to be introduced and established at sites that experience low winter water temperatures. Exposure to high concentrations of ammonium and other organic nutrients from shellfish excretory products may even be a benefit to *P. lima* populations. Carlson and Tindall (1985) noted that *P. lima* is associated with natural habitats characterized by high nitrogen concentrations. Bauder et al. (unpubl. data) observed that batch cultures of *P. lima* grew well in enriched natural seawater (K-medium) with augmented ammonium concentrations as high as 300 μM.

The evidence provided in this study of *P. lima* viability following gut passage in scallops, coupled with the broad ecophysiological tolerances of this dinoflagellate strongly suggest that *P. lima* cells released into the waters of a shellfish aquaculture site via fecal deposition from transferred stock would have a high probability of long-term survival and growth.

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IMMUNOLOGICAL RECOGNITION OF MARINE BIVALVE LARVAE FROM PLANKTON SAMPLES

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ABSTRACT This study concerns the development and use of an immunological method for rapid identification of bivalve larvae in plankton samples. Protein extracts from larvae and adults of various bivalves were prepared and analyzed according to molecular biology techniques (electrophoresis and transfer) for the purpose of species identification. Protein extracts of larvae and adults of *Pecten maximus* induced the production of polyclonal rabbit antibody directed against this scallop. The specificity of these sera was tested by an immunological transfer method (Western blotting). The antibodies selected were used directly on plankton samples. The antigen–antibody pair and, thus, the larvae of *P. maximus* were revealed by secondary markers (colorimetric, fluorescent, and magnetic). The use of such markers is considered for routine work, especially in shellfish farming and marine ecology.

KEY WORDS: Bivalve larvae, antibody, protein recognition

INTRODUCTION

Most benthic marine invertebrates (e.g., the scallop *Pecten maximus*) have a life cycle divided into two distinct stages, benthic for adults and planktonic for larvae, which are closely related in terms of shellfish farming and management. Although most macroscopic species of marine invertebrates have been thoroughly studied, their microscopic stages have often been ignored, particularly because of a lack of suitable "tools" and materials to study organisms under 250 μm in size living in three-dimensional (3-D) space.

Several methods have been used to identify bivalve larvae from plankton samples. One, based on analysis of prodissoconch shape, is applicable only to such families as Anomiidae and Ostreidae (Chanley and Andrews 1971, Le Pennec 1978, Loosanoff et al. 1966). Another limited to the study of hinge characteristics (Rees 1950) was subsequently used with scanning electron microscopy (SEM), enabling different species of bivalves to be reared in the laboratory, particularly during larval and postlarval periods (Le Pennec 1978, Lutz et al. 1982). Unfortunately, this method is time-consuming and difficult to implement, especially for investigation of young stages of bivalve larvae in plankton. Herrera and Cordoba (1981) used immunochemistry to identify bivalve species and suggested that this technique could be applied to taxonomic classification of these organisms in their early stages of development.

In general, immunological techniques allow precise recognition of certain antigens, so that the identification of antigens specific for a given species is possible. This method has not only been applied classically to the medical diagnosis of such pathogenic agents as bacteria and viruses, but also in ecology to determine the diets of different marine vertebrates (Pierce et al. 1990). In fact, the suggestion of Herrera and Cordoba (1981) was not further pursued until the beginning of the 1990s, when Hu et al. (1992) identified bivalve larvae (*Crassostrea gigas*) using protein separation by electrophoresis. A year later, Demers et al. (1993) developed an immunological technique based on monoclonal antibodies to detect the larvae of Pectinidae (*Placopecten magellanicus*) in the sea. Unfortunately, the data provided by these authors were imprecise, which led us to design a new immunological protocol, but not based on monoclonal antibodies. Our basic notion is that molecular modifications probably occur during the different stages of larval life and may block the binding of monoclonal antibodies to their epitope. To avoid this phenomenon, it is preferable to multiply the number of epitope recognized. Thus, our purpose was to develop an immunolabeling process based on the use of polyclonal antibodies that could recognize bivalve larvae in the wild.

MATERIALS AND METHODS

Larval Samples from Hatcheries

The preparation of monospecific antigens to produce "polyclonal" antibodies required the use of batches of larvae of the same species produced by stockbreeding. Several species from three different families (Pectinidae, Ostreidae, and Veneridae) were available. The larvae were collected directly in rearing tanks, washed three times with phosphate-buffered saline (PBS), freeze-dried for 3 h in a Maxi-Dry Coldfinger (FTS ® System Inc. Model FD-4.5-90) and then stored at − 80 °C. *P. maximus* larvae were obtained from a private fishermen's cooperative at Tinduff (Brittany, France) and from the IFREMER experimental bivalve hatchery in Argenton (Brittany, France); *Ostrea edulis* larvae from the IFREMER experimental genetics laboratory at La Tremblade (Charente-Maritime, France); *C. gigas* larvae from the IFREMER hatchery in Argenton and from SATMAR (Socété Atlantique de Mariculture, Gatteville-Phare, Normandy, France); and *Tapes decussatus* larvae from SATMAR and from Tinamenor SA Cantabrica (Spain). All larval stages were represented, from the D-shaped one to pediveliger.

Plankton Samples

Plankton samples were collected in the Bay of Brest using a high-discharge submersible pump, as described by Tremblay and Sinclair (1990). Seawater flowed downward through a 25-cm diameter sieve composed of two nets (the upper one with 300-μm mesh to remove the largest elements, and the lower one with

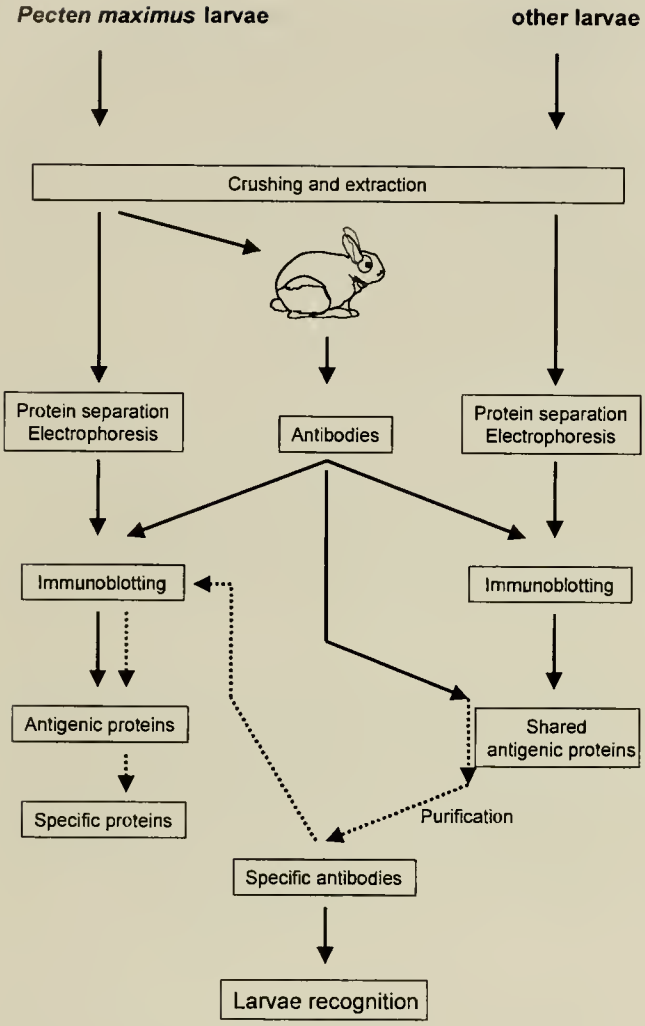

Figure 1. Protocol.

50-μm mesh to retain the plankton fraction likely to contain bivalve larvae). The mesh sizes were suitable for trapping most of the largest bivalve larvae during pelagic development in experimental conditions. Once collected, the plankton was quickly washed with distilled water and stored at 4 °C in 50 mL of 70 ° alcohol.

Adult Bivalves

Thirty or more 3-year old individuals of *P. maximus* and *C. gigas* were collected by scuba diving in the Bay of Brest for subsequent protein extraction.

Protein Extraction

Protein extracts were prepared for electrophoretic analysis and immunisation of New Zealand rabbits. Except for the crushing method, the same protocol was applied to larvae and adults. Larvae were either sonicated or processed with a French press (Bioblock Scientific G 43221); whereas, adults required a precrushing step to break fibres before sonication or French press extraction.

Protocol for Larvae

1. Ultrasonic extraction. Larvae were progressively thawed from – 80 °C in a cold room to 4 °C and then covered with 10 mL of phenol and 10 mL of distilled water before sonication at 50 Hz with a Branson sonifier. To avoid heating, they were subjected to 10 1-second sonication sequences and then placed momentarily (a few seconds) in an ice bath before the next sonication series. This procedure was repeated 12 times to crush most of the shells and suspend the organic matter. The entire solution was stirred for 15 min in a water bath maintained at 68 °C and then cooled to below 10 °C in an ice bath. After centrifugation for 45 min at 4,000 × *g* (4 °C), the pellet was suspended in 4 mL of water at 0.2% in sodium dodecyl sulphate (SDS) containing 1 mmol of phenylmethane sulphonylfluoride (PMSF) and ethylenediamine tetraacetate (EDTA) (proteolysis inhibitors). This solution was dialysed for 2 days in PBS 7.2 containing 0.02% sodium azide, homogenized with a vortex in 8 mL of cold methanol at 0 °C, 4 mL of chloroform and 3 mL of distilled water and then centrifuged for 15 min. at 4,000 × *g*. Once the aqueous phase had been removed, the protein interface was collected and rinsed with 2 mL of distilled water before centrifugation for 15 min. at 4,000 °× *g*. The resulting pellet was dissolved in 2 mL of water before the next centrifugation. This procedure was repeated three times. The final pellet was dissolved in 5 mL of distilled water with 0.2% SDS and 1 mmol PMSF and EDTA to constitute the larval protein extract.

2. French press extraction. The French press was used only to prepare larval extracts. Two millilitres of freeze-dried larvae were suspended in 15 mL of a chloroform:methanol mixture (2 v:1 v), to which 5 mL of distilled water were added before the final solution was poured into the previously cooled 50-mL cylinder of the French press. After 16 metric tons of pressure were applied by a hydraulic press for at least 2 min, the liquid was stirred for 2 min at room temperature and centrifuged for 15 min at 4,000 × *g* at 4 °C. As noted above, the protein extract was contained in the interface layer and required further washing with water.

Protocol for Adults

Because of the large amount of organic matter studied in adult as compared to larval tissues, the tools used for preparation of extracts differed. The tissues were mixed for 2 min at full speed with a kitchen mixer (Moulinex), and the resulting homogenates were treated as for larvae. In the case of scallops, some specific extracts (muscle, mantle, gill, gonad, and digestive gland) were also prepared.

Protein Titration

Protein concentration was determined by a protein-dye binding assay with bovine serum albumin (BSA) as standard (Protein Assay and DC Protein Assay kits, Biorad, ref. 500-0001 and 500-0111, respectively).

Electrophoresis and Blotting

The various protein extracts were partitioned by SDS-polyacrylamide gel electrophoresis (PAGE) (Laemmli 1970). The blotting technique applied was initially designed by Towbin et al. (1979), improved by Kyhse-Andersen (1984), who used a semidry system, and finally modified by Gitton et al. (1992).

Electrophoresis

Aliquots (5 μg) of bivalve proteins were diluted in sample buffer (1:4, v:v) containing 5.5% SDS, 5.5% glycerol, and 1.7% bromophenol blue in 0.5 M Tris, pH 6.8, and then heated for 3 min at 95 °C and subjected to SDS-PAGE in PROTEAN II-type tanks

(Biorad) using the discontinuous buffer system of Laemmli (1970). An optimal acrylamide concentration of 12.5% was selected for the gel. A discontinuous buffer system under electric feed monitored by a Biorad generator (Power-Pac 3000, software version 3.27) was used. Power was constant (17 W), maximum voltage never exceeded 400 V, and the ammeter was set at 100 mA. The gels were stained with Coomassie brilliant blue R-250 O'Farrell.

Determination of molecular mass. The molecular mass of the different proteins was estimated from known standards (Biorad). The method chosen corresponded to a degree 3 polynomial regression model designed from Molecular Analyst software. This model was efficient in describing protein migration in a gel of constant concentration.

Blotting

Electrophoretic profiles were transferred onto nitrocellulose sheets (0.45 μm pore size, Biorad) with a semidry system (Trans-Blot SD Biorad) using Tris buffer (pH 9.2, 48 mmol Tris, 39 mmol glycine, 1.3 mmol SDS, 20% methanol). The nitrocellulose was rinsed out for 5 min in Tris-buffered saline (TBS, pH 7.5) and then blocked for 30 min in TBS-3% gelatine (w:v). Further rinsing with TBS and Tween 20 (TTBS) at 0.05% was necessary, and the nitrocellulose was subjected overnight to the action of rabbit serum at the appropriate dilution (1:2,000) in TTBS at 1% gelatine (w:v). Two additional rinsing in TTBS and one in TBS were necessary to remove unbound antibodies before the marked antiglobulin was labeled with alkaline phosphatase. The blot was then incubated with anti-rabbit IgG antibodies previously conjugated with alkaline phosphatase at a 1:3,000 dilution (v:v) in TTBS-1% gelatine (w:v) and maintained for 30 min in the previous solution. The nitrocellulose was steeped twice in TTBS (5 min) and once in TBS (5 min) and then soaked in Biorad buffer containing nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate. The sheet was finally steeped for 10 min in distilled water. All these steps were carried out at room temperature on an orbital shaker.

Antibody Preparation

Two "primary polyclonal" sera were prepared: one antilarval serum (LPm) and one antiadult serum, (Apm). Rabbit antisera were directed against *P. maximus* extracts. Each serum was prepared using two immunized rabbits. Five immunizations per animal were performed by the subcutaneous route in the back of the rabbit. Each 1-mL dose consisted of one volume of protein solution (900 μg of protein antigens in 500 μL of extract) plus one volume of Freund adjuvant. The first immunization was enhanced by complete adjuvant and the following ones by incomplete adjuvant. The second immunization was performed 2 weeks after the first, and the last three at 1-week intervals. The final serum was collected 5 days after the last injection.

Antibody Purification

The entire antiscallop larval serum (LPm) was consumed in 1 day under stirring at 4 °C against an extract of adult *C. gigas*. The purified serum or $LPm_{consumed}$ obtained in supernatant after 45-min centrifugation of LPm-*C. gigas* extract at 4,000 × *g* at 4 °C. Compared to the dilution ratio used for blotting, $LPm_{consumed}$ was less diluted (1:400).

Larval Recognition by Antibodies

All organisms contained in plankton samples were washed twice for 5 min in PBS under soft stirring, incubated for 1 h with 1% glutaraldehyde in PBS, washed twice for 5 min in PBS, incubated 2 to 15 min in 0.2% Triton X-100 in PBS, and washed again for 5 min with PBS. To reduce nonspecific staining, organisms were incubated for 30 min with 3% hydrolyzed gelatine in PBS (w:v). After two washes in PBS (5 min), larvae were incubated overnight in $LPm_{consumed}$ (diluted 1:400 in PBS), rinsed twice with PBS and incubated for 30 min with goat antirabbit IgG labeled: (1) with alkaline phosphatase (Biorad); or (2) fluorescein-isothiocyanate (Interterchim); or (3) with magnetic beads (Dynabeads M 280-Dynal®, 2.8 μm diameter). Once washed with PBS, all organisms were ready for observation, except those tagged with alkaline phosphatase (this stain required a substrate to induce blue color; i.e., nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate in Tris buffer, pH 9.0).

All organisms were observed with a universal epifluorescent microscope (Zeiss Orthoplan model) using a 200-W mercury lamp with an appropriate FITC filter. Photos of plankton samples were taken with a mix of transmitted and epifluorescent light. The mercury lamp was turned off to observe the two nonfluorescent-stained samples.

RESULTS

Protein Titration

The protein-dye binding assay kit (Bradford 1976, Lowry et al. 1951) was used to evaluate extraction yield. The concentrations in larval protein measured in *P. maximus* from Argenton and Tinduff were 5 and 1 mg/mL^{-1} respectively; whereas, in Ostreidae, they accounted for 1 mg/mL^{-1} in *C. gigas* and 0.4 mg/mL^{-1} in *O. edulis*. The level in *T. decussatus* was 1.7 mg/mL^{-1}.

Results were similar, regardless of the extraction method used (ultrasound or the French press), but the French press protocol was shorter and, thus, time-saving.

Electrophoresis

After protein determination, the different extracts (larvae and adults from *P. maximus* and *C. gigas*; larvae from *O. edulis* and *T. decussatus*) were subjected to 12.5% SDS-PAGE with Coomassie brilliant blue staining. Adult extracts displayed several bands with similarities indicative of a common profile for the tissues of these two species (*P. maximus* and *C. gigas*). However, the sequence and thickness of the different bands depended on each species and were characteristic of them. Although common strips were found between larval and adult extracts, there were some significant differences within a given species.

Comparative analysis of electrophoretic profiles from the whole set of extracts highlighted several bands, each characteristic of a single bivalve species. The 87 kilodalton (kDa) band corresponded to adult *P. maximus* the 30 kDa one to *P. maximus* larvae, and the 70 and 32 kDa bands, respectively, to *Ostreidae* and *T. decussatus*. However, the protein bands, though specific, were not necessarily immunogenic.

Blotting

Rabbit antibodies on blots, which corresponded to all the electrophoretic profiles after transfer, revealed the immunological response. Serum LPm (Fig. 2A) and APm (Fig. 2B) were applied, respectively, onto the blot at a dilution of 1:2,000.

After full immunization, rabbit antibodies were able to recognize numerous protein antigens. LPm, like APm (both produced

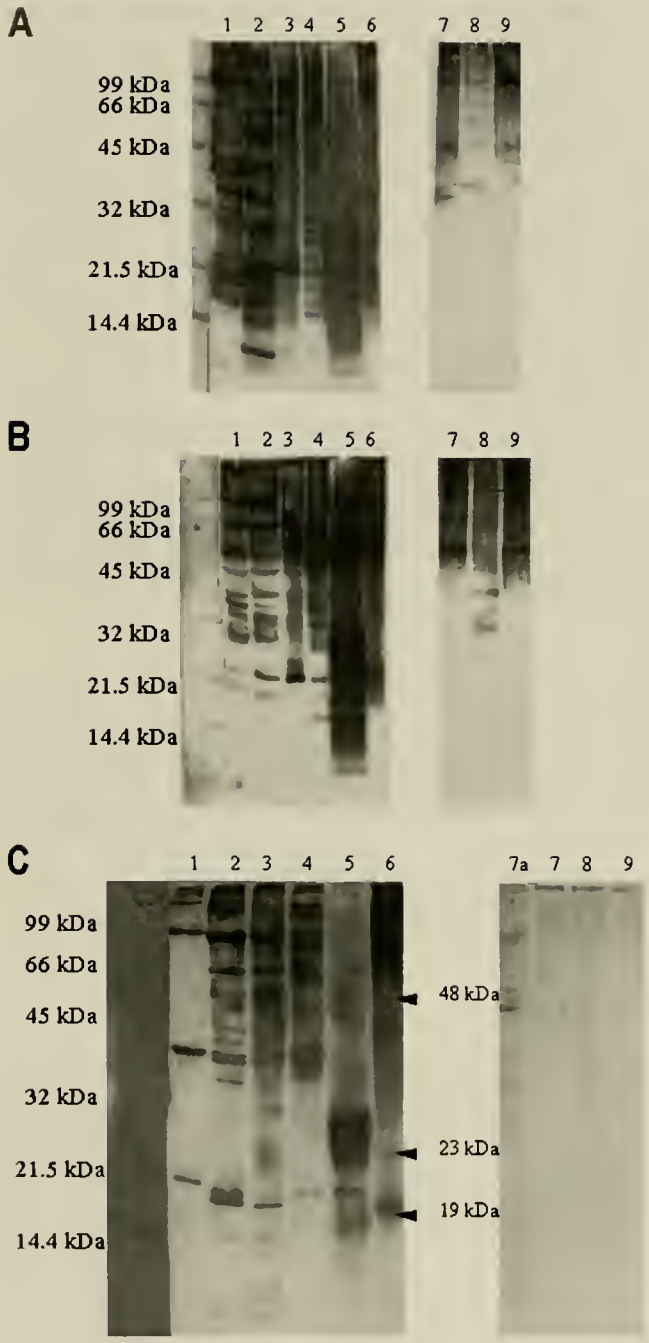

Figure 2. Western blotting of proteins obtained from *P. maximus*, *C. gigas*, *R. decussatus*, *O. edulis*. (A) revealed by APm. (B) revealed by LPm. (C) revealed by LPm consumed. Lanes : 1 to 6 extracts of P. maximus. 1, Adult muscle; 2, Adult mantle; 3, Adult gill; 4, Adult gonad; 5, Adult digestive gland; 6, Larvae. 7a, Adult *C. gigas*; 7, Larvae *C. gigas*; 8, Larvae *R. decussatus*; 9, Larvae *O. edulis*.Molecular weight is indicated as kilo-Dalton for each lane.

from *P. maximus* extracts), reacted with the other bivalve species investigated. Polyclonal rabbit sera allowed us to detect antigenic proteins in scallop extracts by immunoblotting and also highlighted several antigenic bands shared in common by scallops on extracts from other species. Each electrophoretic profile was compared with the two immunoblots, allowing identification of the antigenic bands on the electrophoretic profile and especially detection of those found only in the extract from the chosen species; that is, *P. maximus* larvae.

Immunostaining intensity was correlated with antiserum sensitivity; whereas, Coomassie blue staining was related to protein abundance.

Immunological Recognition of *P. maximus* Extracts

Investigation of the immunological recognition of *P. maximus* extracts showed that LPm serum reacted more specifically with extracts from *P. maximus* larvae than with those from adults; whereas, the reaction with APm was stronger against adults than larvae. The effect of two antilarva and antiadult sera on an adult extract of scallops differed significantly, especially for antigens below 21.5 kDa. This molecular mass constituted a limit below which homology was no longer apparent. For each blot, homologous reaction was always greater than heterologous reaction.

Immunological Recognition of all Other Bivalve Extracts

The antiadult serum (Apm) reacted preferably with *C. gigas* adults The response against larval extracts was evenly distributed along the strips, but did not allow clear identification of the different bands, except for the 100-kDa band, which occurred in all bivalve extracts. This serum did not distinguish between the different extracts. For each of the four extracts; that is, *C. gigas* larvae and adults, *O. edulis* larvae and *T. decussatus* larvae, the antilarval serum (LPm) did not "react" below 16 kDa. Once again, the resulting immunoblotting profiles were characteristic of the species represented. However, LPm serum recognized bands probably shared by all species investigated. A comparison of profiles with that corresponding to the larval extract from *P. maximus* enabled us to identify two specific bands at 48 and 23 kDa respectively.

For the two sera, analysis of the sequences of all bands constituting each profile indicated that each protein extract was specific and that the sera used to recognize them had a strong affinity for the extract, which had induced their elaboration. However, the polymorphism of these sera was too great to allow their direct use as a recognition marker.

The Use of $LPm_{consumed}$ Serum

Because the antigens were generally common to several species and especially to the extract from adult *C. gigas*, antibodies of LPm antiserum were adsorbed on a mixture of *C. gigas* tissues to remove all the antibodies shared in common by *C. gigas* adults and *P. maximus* larvae. The resulting consumed serum, $LPm_{consumed}$ (Fig. 2C), did not react with the extract of *C. gigas* adults. This procedure allowed the capture of antibodies common to this species of bivalve. Among the larval extracts, only that of *P. maximus* was identified by the adsorbed serum. This purified serum confirmed the presence of specific *P. maximus* larval bands at 48 and 23 kDa and identified a new one at 19 kDa.

In Vivo Labeling Process

Indirect antibody staining was performed on the $LPm_{consumed}$ serum (Fig. 3) using three different types of anti-antibodies labeled with alkaline phosphatase (ALP) (Fig. 3A), FITC (Fig. 3B, D) or magnetic beads (Dynal®)(Fig. 3C).

With the first staining procedure, labeling was especially concentrated around the shell on the mantle edge. The FITC antiglobulin allowed us to localize a scallop veliger from a plankton sample

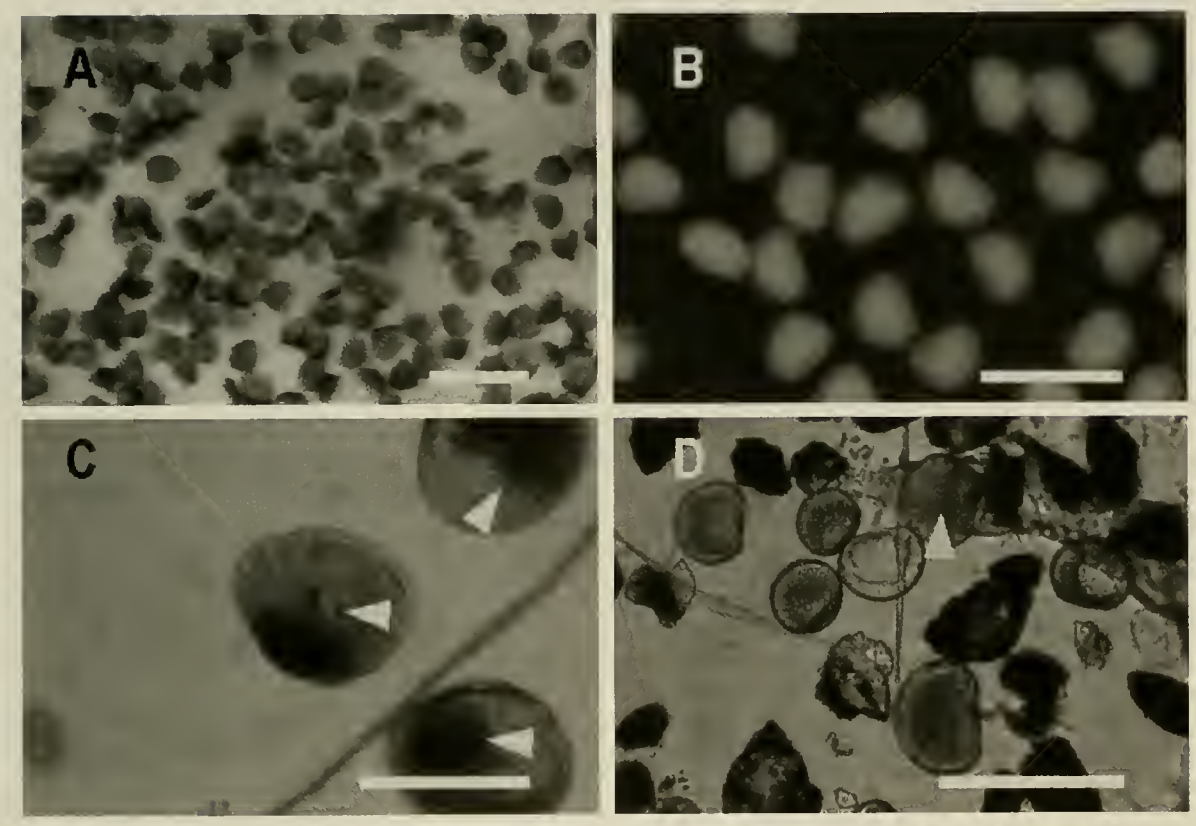

Figure 3. Larval immunostaining. (A) Scallops larvae revealed with Alkaline Phosphatase; (B) Scallops larvae revealed by FITC; (C) Scallops larvae revealed by magnetic beads; (D) FITC labeled scallop larva revealed in a plankton sample. Scale bar = 100 μm.

under epifluorescent microscopy, using mixed fluorescence wavelengths (Fig. 3D) and visible ranges, as suggested by Demers et al. (1993). $LPm_{consumed}$ recognized both D larvae (Fig. 3B) and the pediveliger. The immunomagnetic marker (Dynabeads M-280) formed a complex with $LPm_{consumed}$ to stain scallop larvae, but did not magnetize them sufficiently to allow total collection by magnetic power. Nevertheless, many larvae were captured in the magnetic field (in Fig. 3C, each black point is an aggregate of many magnetic beads).

DISCUSSION

Only monoclonal or polyclonal antibodies can be used to stain bivalve larvae by an immunological technique. The formers have high specificity dependent on selective binding to only one epitope. Demers et al. (1993) tested this technique and found variability in stain intensity within the same batch of larvae. One possible explanation for these results was a differential degradation of antigenic structures attributable to transport and conservation conditions. A second possibility was that antigen expression changed naturally as a result of physical and (mainly) physiological conditions during larval development, as previously reported by Boreham and Ohiagu (1978), Feller (1986), Wang *et al.* (1992), Westin (1972), etc. To decrease the variability in staining intensity, Demers *et al.* (1993) used a pool of monoclonal antibodies selected among three specific lines.

Instead of applying a pool of monoclonal antibodies, we used the rabbit for preparation of a polyclonal serum, which processes several antibodies against all the antigens in a preparation and, unlike monoclonal antibodies, produces stable multivalent interactions conducive to high affinity. Among substances likely to be immunogenic, proteins are most often used, and our extraction procedures sought to obtain a good protein immunogen. Two grinding methods were tested (results not shown). The French press proved not only more efficient for protein extraction from bivalve larvae, but also saved time by avoiding a first extraction with a mixture of water and phenol. In addition, the simplicity and efficiency of this method reduced solvent consumption and larval cost while lowering inhalation risk in a small enclosure. In any event, the electrophoretic profiles were similar with both methods, and analysis identified the bands characteristic of the proteins contained in each extract and thus in each bivalve species.

Extraction yields were always evaluated using the protein-dye-binding assay kit. Protein amounts varied with larvae batches, but were not correlated with the investigated species. Adult extracts presented no difficulties. Regardless of the extraction yields, electrophoretic profiles were characteristic of a single species. When protein levels were below 1 mg/mL^{-1} at the end of extraction for larvae, only the readability of the profile was affected (blurred bands), suggesting a possible correlation between low protein content and blurred profiles. As extractions were always carried out in the same environmental conditions and electrophoresis was performed on the same amount of proteins, the only possible explanation for this yield reduction related to the larvae themselves. In fact, all came from stock farming. Although their shell morphology criteria were fine, their true health conditions remained unknown. Unfortunately, no information was available on the settling capacity of each batch of larvae, which might have allowed the quality of the electrophoretic profile to be related to postlarval settlement (the only true index of larval physiological health).

As noted above, any protein extract from a given bivalve species was liable to produce a polyclonal serum with a specificity sufficient to differentiate one extract from another by immunoblotting. LPm and APm enabled us to distinguish the immunological bands characteristic of a particular *P. maximus* extract, and molecular analysis software allowed us to highlight the succession of bands characteristic of scallops. Extracts from adults and larvae shared several bands. However, this approach neglected the importance of using larval extract as antigens to induce specific "anti-larval" antibodies. Although the specificity of our LPm serum was sufficient to recognize scallop larval extract among several others, it was unable to recognize scallop larvae in plankton (unpublished data).

Two possible procedures were considered to enhance the specificity of our first sera: the capture of specific antibodies by chromatography and the removal of nonspecific antibodies by depletion. The second solution proved easier to implement. To limit potential cross reaction, an attempt was made to purify LPm before subjection to heterologous molecules. The recognized antigen allowed the formation of a complex with antibodies that settled down. After centrifugation, the pellet was easily removed and discarded. Thanks to this technique, a new version of the LPm serum ($LPm_{consumed}$) was obtained, which in immunoblotting tests recognized scallop extracts (mainly from larvae). There were no further cross reactions with larval extracts from other species. The absence of a heterologous reaction allowed us to use this serum for larva labeling. Antibody binding was revealed by three different techniques. First, staining with alkaline phosphatase localized the area of antibody binding near the mantle. Like Demers et al. (1993), we found that no staining disorder was apparent because of shell closure. Alkaline phosphatase was not selective, because it revealed the enzyme bound to the antibody as well as the endogenous enzyme. Revelation of the latter caused background noise in plankton samples (some controls were made on batches of larvae with negative antibodies or with direct incubation in AP substrate), which affected the interpretation of bivalve larval detection. Fortunately, this inconvenience can be avoided if revelation time for the enzyme does not exceed 15 min.

Fluorescent staining gave better results (Fig. 3B, D), allowing scallop larvae to be identified in plankton samples. Because the wavelength used to induce antibody fluorescence sometimes makes phytoplankton fluorescent, some adjustments will be required before a flow cytometer can be used to automate the counting of bivalve larvae. Staining could be improved by replacing the fluorescent marker with another one in order to change the excitation wavelength.

Magnetic beads have already been used in marine biology for isolation of the toxic dinoflagellate *Alexandrium fundyense* from preserved seawater (Aguilera et al. 1996). In our study, beads were found within the larvae. Although their magnetization was too weak for efficient separation of all scallop larvae from plankton, bigger bright-colored magnetic beads could allow the separation and recognition of scallop larva even by an inexperienced researcher. All three of these staining methods allowed rapid identification of bivalve larvae among the whole plankton community.

In conclusion, this first approach to recognition of bivalve larvae indicates the possible benefit of the methods considered here. Electrophoresis revealed immunogenic and specific proteins in several bivalve species. Once purified, such proteins will be able to induce new polyclonal sera endowed with higher capabilities, as recommended by Knudsen (1985) and Diano et al. (1987).

This approach reduces the time required for sorting out and identifying scallop larvae in plankton samples. The use of polyclonal antibodies to stain the larvae extracted from our samples seems promising, and it is likely that this method, after a few adjustments, can be applied in ecological programs or for shellfish farming.

The major interest of this technique is species-specific identification to distinguish close species such as *Pecten maximus*, *Aequipecten opercularis* and *Chlamys varia* in the Bay of Brest, or *Placopecten magellanicus* and *Chlamys islandica* in the Gulf of St. Lawrence in Canada. However, many more families could be treated as well. The use of an immunoassay for identification of marine larval species could save precious time and allow automated identification and measurement of larvae. All these improvements could be useful in shellfish farming to monitor the settlement of postlarvae and determine the best period for setting up a collector.

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TECHNICAL PAPERS

Presented at

INTERNATIONAL CONFERENCE ON SHELLFISH RESTORATION

Hilton Head Island, South Carolina

November 18–21, 1998

Journal of Shellfish Research, Vol. 19, No. 1, 334, 2000.

SELECTED PAPERS FROM THE

1998 INTERNATIONAL CONFERENCE ON SHELLFISH RESTORATION:

"FORGING PARTNERSHIPS TO IMPROVE THE HEALTH OF COASTAL ECOSYSTEMS THROUGH SHELLFISH RESTORATION"

Throughout the world there is a growing commitment to the restoration of degraded coastal ecosystems. Political pressure by shareholders in the future of the world's coastal areas has resulted in renewed interest in preserving and enhancing coastal resources at all levels of government. At the local level many volunteer organizations have developed successful programs to identify problem areas, recommend improvements and monitor progress.

The Second International Conference on Shellfish Restoration (ICSR) was held on Hilton Head Island, South Carolina, U.S.A. on November 18–21, 1998. ICSR provides an opportunity for government officials, resource managers, users, and residents to discuss approaches to restore coastal shellfish ecosystems through remediation and pollution abatement, habitat restoration and stock enhancement. Case studies of successful projects are presented, with opportunities for roundtable discussions.

The first ICSR event, held in 1996 was extremely successful. More than 200 participants from ten countries joined together to learn about and discuss innovative management, ecological, and social approaches to restore degraded shellfish habitat and improve coastal ecosystem health. ICSR has been so successful that a European version of ICSR was born—ICSR'99 was organized and held last year in Cork, Ireland.

A unique feature of ICSR is the diversity of individuals who participate. The opportunity for internationally recognized restoration experts to interact with local town planning officials does not occur often enough. ICSR provides that forum and also the building blocks for such interactions to occur in the future.

The papers that follow are representative of the diversity of the topics and individuals that participated in ICSR'98. We are grateful to the many conference sponsors for their support, the members of the ICSR Steering and Program Committees for their time and energy, and to the Journal of Shellfish Research and its editor, Sandy Shumway, for publishing these contributions. We also wish to thank Anne B. Miller for serving as our Technical Editor. Thank you all.

From the ICSR'98 Co-Chairs: Dorothy Leonard, NOAA-NMFS, M. Richard DeVoe, Elaine L. Knight, and Linda Blackwell, S.C. Sea Grant Consortium, and William Rickards, Virginia Sea Grant College Program.

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RESTORING THE OYSTER REEF COMMUNITIES IN THE CHESAPEAKE BAY: A COMMENTARY

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ABSTRACT Restoration of the oyster *Crassostrea virginica* resource to the Chesapeake Bay is a widely supported goal. This manuscript explores the questions of why, how, and in what time frame this should be attempted. Restoration goals based simply on support of a commercial fishery fail to address the role of the oyster as a cornerstone species within the Chesapeake Bay and should only be considered in the context of a long-term sustainable fishery exploitation. The argument is proffered that a restored resource sustaining a fishery at the historical harvest level is unrealistic, because: (1) harvest probably exceeded biological production for much of the recorded history of exploitation; and (2) maximum production, a desired end for fishery support, occurs at approximately half the maximum (virgin, unexploited) biomass, and, thus, can only be achieved with disruption of the virgin complex community structure. Thus, the direct harvest economic value of a fishery based on a restored resource will not reach historical levels if there is an accompanying goal of long-term community development that is self-sustaining in the absence of restoration effort. The role of the oyster as a cornerstone organism and the pivotal link in benthic-pelagic coupling is examined in the context of current and projected watershed management problems, including agricultural and urban development with associated nutrient and sediment erosion issues, in the entire Chesapeake Bay watershed. Restoration efforts to date have focused on rebuilding three-dimensional reef structures, often with subsequent oyster broodstock enhancement, in predominantly small estuaries with retentive circulation to provide demonstration of increased resultant recruitment. Such examples are used to increase public awareness of the success of restoration processes and increase long-term participation in such programs by schools, nonprofit and civic organizations, and commercial and recreational fishing groups.

KEY WORDS: oysters, *Crassostrea virginica*, Chesapeake Bay, reefs, restoration, watershed, management, benthic-pelagic coupling

DEFINING THE PROBLEM, PART I: BIOLOGY, ECONOMICS, PERCEPTION, AND TIME FRAMES

The Chesapeake Bay has a history related to the eastern oyster *Crassostrea virginica*. Much of the biology of the bay over the past 10,000 years is arguably dependent on the reef-forming habit of this cornerstone species. Oysters were an important food source to pre-Colonial native populations, were quickly recognized for their value after Colonial settlement, became the center of a national and international trade before the end of the 19th century, and remained a substantial component of the Middle Atlantic economy through the first six decades of the 20th century. The past four decades have been marked by the appearance and continued destructive effects of two disease vectors, *Haplosporidium nelsoni*, commonly known as MSX, and *Perkinsus marinus*, commonly known as Dermo, in the higher salinity regions of the bay.

When considered together with the cumulative effect of many decades of overfishing and environmental decay, the result is a sadly depleted oyster resource in the Chesapeake Bay. Although consensus is growing that attempted restoration of this resource is a noble and worthwhile cause, the task before us is to ask why, how, and in what time frame this should be attempted.

Given that the oyster has long supported a commercial fishery in the Chesapeake Bay, a logical first question is "Should the revitalization of the oyster fishery be the prime motivation for restoration of the oyster populations in the bay?" Such a question has a number of inherent qualifiers. Fisheries utilize a biological resource to optimize or maximize economic or societal return. Restoration of the resource for this prime purpose would be in a form that optimizes harvest over a defined time frame—a form that may not, as is discussed later, be considered best for optimizing ecological complexity and stability. Economies have time horizons of importance, thus any restoration effort must respect and be responsive to this time frame. The societal component must be equally addressed in that restoration to enhance an economic contribution to a thriving economy must be responsive in a politically expedient time frame; that is, efforts must create a strong public perception of improvement in the face of multiple competing needs for public funding. Economy, perception, and time frame, in addition to biology, become important factors in setting fishery restoration goals.

What might be reasonable goals for a fishery-driven restoration program? The recent and current oyster fishery in the Chesapeake Bay has several components. These must be distinguished from the oyster industry, which includes processing of oysters originating in regions other than the bay. In Maryland, there currently exists an active public fishery prosecuted by watermen who purchase licenses to harvest oysters from resources in regions held in public trust by the State of Maryland. The harvest from this fishery typically exceeds its Virginia counterpart by a very substantial amount. However, this harvest is "underwritten" both by substantial public funds and by the continuing effort by the Maryland Department of Natural Resources to plant shell substrate in selected regions in advance of seasonal oyster settlement (spatfall) and to move the resultant "seed" to regions for optimal growth in the face of potential disease pressure. This program is arguably very responsive to a fishery need; the long-term issue of resource restoration is not a prime mission of the program. A similar program of shell deployment and subsequent "seed" movement on grounds retained in public trust has also been pursued in Virginia. The incremental impacts of disease have reduced the effectiveness of the Virginia program in supporting a continuing industry, and current landings from the fishery are at an all-time low. As in Maryland, the focus of this "plant and move seed" program has been short term, with

no statement on long-term restoration. Virginia also allows leasing of "suboptimal" bottom adjacent to public grounds. These regions sustain a very substantial fishery harvest essentially in grow-out of "seed" oysters but are operated on a put-and-take basis with a 2–3 year growth period. Again, these are strictly for-profit operations by private individuals or corporations with no restoration goal (see Haven et al. 1981a,b). Such efforts have all but disappeared in the past decade as a result of the continued incidence of disease. The losses accompanying the fishery's decline since the major onset of disease have had a subtle societal impact that has generated considerable public debate and, in some instances, sympathy. Commercial fishermen are among the few remaining "hunter-gatherers" in modern society, and their visible demise in the Chesapeake Bay oyster fisheries is viewed (perhaps unrealistically) as a loss of individuals who operate with large amounts of personal freedom in a society that pays little attention to that same personal freedom. A reasonable goal from an economic position would be the restoration of a fishery resource to support a predisease level of harvest, typically several millions of bushels per year for Maryland and Virginia combined, with some enhancement of the societal role supported by the fishery.

Is a fishery-driven restoration to sustain a predisease level of harvest a reasonable goal for ecological restoration? Arguably, no. An examination of the historical fishery harvest finds that the harvest was much greater before to the turn of the century. The combined harvest of oysters in 1865 by Maryland and Virginia alone was approximately 17 million bushels (Hargis and Haven 1988)—enough oysters to bury a football field to the depth of 656 feet! This is an astonishing amount given the primitive dredging and tonging techniques employed, but it illustrates simply the level of fishing pressure employed in the latter half of the 19th century. We know from the works of Ingersoll (1881), Brookes (1891), and others that a century ago strong concerns about overfishing and its eventual impact were expressed to regulatory bodies. Although these concerns stimulated a limited regulatory effort, and the surveys of Winslow in Maryland and Baylor (1894) in Virginia to define the extent of the public resource, the comments did little to abate the revisiting of the "tragedy of the commons." The important issue to this commentary, however, is that the enormous removals of oysters had proportionate impact on the biology of the oyster in the bay. Neither as part of the process of oyster harvesting nor as part of the discussion (minimal for much of the time) on resource management was a formal assessment of stock size or estimate of productivity ever made. However, the fundamental understanding of the importance of these processes was already central to the discussion of marine finfish stocks on both sides of the Atlantic before the turn of the century, as demonstrated by the work of Spencer Baird, G.O. Sars, and their peers. Although very large and obviously old oysters were still abundant in the bay during the heyday (1860s) of harvest (de Broca 1865), we also know from navigation charts prepared by the U.S. Navy before the turn of the century, that three-dimensional oyster reef structures were exposed only at low tide in many regions of the bay. These reefs gradually became permanently subtidal with continued wholesale mining of the resource for both food and industrial (chicken grit to limestone to road surfacing material) purposes. Indeed, gradual submergence of the reefs could not be ascribed to sea level rise!

Proceeding further back in time, we move from the period of highest harvest in the latter half of the 19th century to the period of Colonial settlement, when intertidal oyster reefs were abundant and notable features of the bay. It is this presettlement era that illustrates the most defensible target for restoration goals. Throughout the preceding discussion there has been frequent mention of fishery harvest, but purposely not of biological production. In a well-managed, economically exploited resource, the harvest does not exceed production. Given the lack of assessment and productivity data, a definitive temporal analysis of the post-Colonial settlement harvest in excess of productivity is not possible. However, we do know that the cumulative result has been the removal in less than 400 years of complex reefs that developed over a 10,000-year period, beginning with the inundation of the bay in the current postglacial warming period.

Acceptance of the tenet that cumulative harvest was clearly in excess of cumulative production places the question of restoration for fishery harvest in a new light. The projection of a restored resource being able to sustain a fishery at the historical harvest level is unrealistic because: (1) harvest probably exceeded biological production for much of the recorded history of exploitation; and (1) maximum production, a desired end for fishery support, occurs at approximately half the maximum (virgin unexploited) biomass (as defined in Applegate et al. 1998, Restrepo et al. 1998) and, thus, can only be achieved with disruption of the virgin complex community structure. Indeed, the direct harvest economic value of a fishery based on a restored resource will not reach historical levels unless there is an accompanying goal of long-term community development that is self-sustaining in the absence of restoration effort. It is, therefore, unreasonable to consider a restoration effort for oyster fishery support purposes alone. This conclusion prompts the question, "If the goal is not just the fishery harvest, what end point should restoration goals seek to achieve?" I argue that oyster restoration should be viewed as the reestablishment of (one of several) cornerstones in an ecosystem.

DEFINING THE PROBLEM, PART 2: A CORNERSTONE IN THE ECOSYSTEM

The reason oysters are the focus of shellfish restoration in Chesapeake Bay is their value as a cornerstone species in the bay. Oysters are a major benthic-pelagic coupler; one that supports a diverse food web in higher trophic levels and, as an added bonus, is the basis of a commercial fishery of secondary importance to the food web structure.

How big is the baywide problem? Enormous. The Chesapeake Bay is 298 km long (185 miles), has a surface area of 8,484 km^2 (3277 sq. miles), and has a volume of 71.5×10^9 m^3 (Cronin and Pritchard 1975). Within this context the biology of the oyster deserves attention. Oysters are gregarious and long-lived (therefore, large) in a pristine environment. Spawning efficiency is maximized by simultaneous gamete release in these dense aggregations (see studies by Levitan 1991, Levitan et al. 1991, 1992 for sessile benthic organisms, sea urchins, in spawning and fertilization efficiency). Individual fecundity increases with size (Thompson et al. 1996 using data from Cox and Mann 1992), so dense aggregations of large animals should be a goal of restoration, because they help provide long-term stability through provision of larval forms. Dense aggregations grow in the third dimension (up) in the presence of adequate food. Multigeneration aggregate settlement creates three-dimensional structure as older animals die but remain as substrate for new recruits to the benthos. Three-dimensional structure would, therefore, seem to be a further defensible goal of restorative efforts.

The trophic role of oysters in the Chesapeake Bay and other similar systems has been well studied; therefore, quantitative arguments can be proffered: (1) to support the level of restoration; and (2) to estimate the trophic impact on both nutrient reduction through grazing and higher trophic production through support of enhanced food chains (see Newell 1988, Baird and Ulanowicz 1989, Ulanowicz and Tuttle 1992, for examples). An examination of these contributions underscores the need to consider oyster restoration not as a singular goal but as a component of a holistic approach to watershed management that includes land use practices and the subsequent impact of riverine input to water column processes throughout the bay and its subestuaries. Water column processes are then to be considered in the context of local habitat and benthos (including oyster reefs), progressing to include resident and seasonally migratory transient macrofauna. The complexity and size of the problem has, fortunately, received much attention. The NOAA and EPA Chesapeake Bay Program databases in addition to those of the U.S. Geological Survey (most of these are now available through the World Wide Web) are replete with useful information to guide the restoration plan. To reiterate, a restoration process must be placed in a time context. The changes in the original watershed from forested to a mix of urban, agriculture, and forest occurred over the period from Colonial times to the present; the projected population growth through 2020 within the watershed, especially the coastal regions of Maryland and Virginia, exceeds projected national growth rates. Increases in the human population within the watershed from the current 14 million to 16–18 million are within reason in this time period. Attempts to plan and control growth within the watershed are and will continue to be both politically charged and difficult to resolve. Unfortunately, historical precedent illustrates a general lack of resolve in this country to limit growth and exploitation effectively. Therefore, land use and runoff issues associated with these projections will raise discussion of freshwater diversion, use, re-use, discharge, groundwater use and contamination, and saltwater intrusion. Every item on this list directly affects nutrient and sediment inputs to the bay and will tax the capabilities of recent amelioration strategies to reduce negative effects.

The biological consequences of increased inputs of nutrient and particulate material to the bay watershed are well understood. Nutrients stimulate productivity in excess of the grazing capacity of the resident filter feeders, notably the benthic filter feeders. Sediment loads that inhibit the filtering process exacerbate the situation. With limited grazing, eutrophication is inevitable. Sediment loads similarly inhibit extension of submerged aquatic vegetation (SAV) by limiting light penetration of the water column. The complex nature of the restoration problem is well illustrated by consideration of a two-species interaction: that of oysters with SAV. On a riverwide scale the presence of multiple reef systems with vertical relief in otherwise open bodies of water, like much of the Chesapeake Bay, reduces fetch and, hence, wind-driven resuspension of particulate material in the water column. The presence of fringing reefs reduces sediment input from shoreline erosion. At a smaller scale, filter feeding by oysters reduces water column loads of sediment and plankton; thereby, increasing light penetration and increasing SAV growth. Bottom stabilization by SAV increases water quality; thereby, encouraging a positive feedback loop to oyster growth. There is nonlinearity in this feedback: when the suspended sediment load increases above a certain level, SAV growth essentially ceases, and the response of the oyster filter-feeding rate to sediment load approximates a parabola. Thus, although publicly stated goals of 40% nutrient reduction in nutrient input are laudable, they must be accompanied by a critical reduction in sediment load to allow SAV growth and the oyster–SAV positive feedback interaction to develop. This multifaceted problem of both elevated nutrients and sediments is notable in areas that once supported abundant oyster populations—the James, York, and Rappahannock rivers, and Pocomoke–Tangier Sound—and are given critical status on current Chesapeake Bay Program and EPA World Wide Web sites. Proceeding above a "simple" two-species interaction, Lenihan and Peterson (1998) underscore the sensitivity of the multispecies interaction on reefs to multiple environmental factors.

The enormousness of the potential restoration effort and its primary goal is easily recognized. Is there a logical recovery protocol? I argue, yes. The unique aspects of the biology of the oyster in the bay that must be exploited to facilitate restoration are known: high density and a three-dimensional structure in a location where filter feeding will not be overwhelmed initially by local water quality conditions. In Virginia, these aspects have been used to guide the choice of location for early restoration efforts. A critical issue from both the biological and political view is the choice of sites. Sites must be selected such that the impact of the effort is visible in a short (months to a small number of year) time frame; that is, the signal from the restoration effort must be "visible" above the natural variability or "noise" in the target system. Thus, there is a need to match scale of effort with goals. Attempting wholesale restoration of large river systems at the outset is clearly not viable for either cost or biological considerations, but there are many smaller parts of candidate systems that are attractive. Using such resources as the Baylor ground maps (1894), natural reef "footprints" have been identified that can be cleaned of remaining oysters and used as a base to build three-dimensional structure.

Under the guidance of the Shellfish Replenishment Program at the Virginia Marine Resources Commission, a reef-based restoration effort was initiated in the Piankatank River in 1993 with construction of a single reef at Palace Bar. No broodstock addition was effected at the site. Construction is described in Bartol and Mann (1997). Since its construction, this site has been studied intensively in terms of oyster recruitment and growth (Bartol and Mann 1997, in press, Mann and Wesson unpublished data), disease progression in recruited oysters (Volety et al. 2000, this issue), and development of associated fish and benthic communities (Harding 1999, Harding and Mann 1999, 2000). A contrasting approach was employed in the Great Wicomico River in 1996 (Southworth and Mann 1998). The success of this effort warrants description as a model for restoration programs. The Great Wicomico River is a small, trap-type estuary on the western shore of the Chesapeake Bay that once supported substantial oyster populations. The combined effects of Tropical Storm Agnes in 1972 and subsequent disease mortalities related to *Perkinsus marinus* and *Haplosporidium nelsoni* essentially eliminated these populations. Oyster broodstock enhancement was initiated in June 1996 by the construction of a three-dimensional intertidal reef with oyster shell, followed by "seeding" of the reef in December 1996 with high densities of large oysters from disease-challenged populations in Pocomoke and Tangier Sound. (In these donor locations, the extant oyster population density is too low to effect reasonable probability of fertilization success and subsequent recruitment.) Calculations of estimated fecundity of the resultant reef population suggested that oyster egg production from this source were within an

order of magnitude of total egg production in the Great Wicomico River before Tropical Storm Agnes. Field studies in 1997 indicated spawning by reef oysters from July through September; whereas, plankton tows recorded oyster larval concentrations as high as 37,362 ± 4,380 larvae/m^3 (on June 23)! Such values are orders of magnitude higher than those typically recorded for Virginia subestuaries of Chesapeake Bay in the past three decades and strongly endorse a premise of aggregating large oysters to increase fertilization efficiency. Drifter studies suggest strong local retention of larvae, a suggestion reinforced by marked increases in local oyster spatfall on both shell string collectors and bottom substrate in comparison to years before 1997. Although disease was evident in the population—*Perkinsus* prevalence increased from 32% in June to 100% in July, and intensity increased from June to September—the effort demonstrated that choosing locations where local circulation promotes larval retention combined with reef construction and broodstock enhancement may provide an accelerated method for oyster population restoration. Following the above observation in the Great Wicomico, two reef sites in the Piankatank have been added as part of the broodstock enhancement program using large oysters collected from high salinity regions of the bay where disease pressure remains high. Similar efforts are underway in two small tributaries of the Potomac River (the Coan and Yeocomico), the Elizabeth River, Pungoteague Creek on the bay side of the Eastern Shore of Virginia, and Lynnhaven Bay on the south shore of the Chesapeake Bay mouth. In addition, reefs of various substrate types have been constructed at Fisherman's Island at the southern tip of the Eastern Shore of Virginia and are the site of continuing intense study by Luckenbach and collaborators based at the Virginia Institute of Marine Science Wachapreague Laboratory.

Although there is a clear generic component to these individual efforts of small reefs in small systems, each site is unique along a salinity cline within Virginia waters. They represent a mosaic of habitat types with differing environmental values in both biology and physical structure. Such unique aspects of each reef system are examined further by Breitburg et al. (2000, this volume). Provision of complex physical habitat structure provides opportunity for recruitment by species other than oysters as demonstrated by Breitburg et al. (1995), Breitburg (1999), Harding and Mann (1999, 2000), Nestlerode and Luckenbach (in press), and Coen and Luckenbach (in press). To date, the progression of increasing species richness and complexity in relation to presence or absence of "seeded" oyster broodstock has not been investigated, although it is reasonable to suggest that the presence of the latter accelerates development of the multitrophic community on and around the reefs.

The problem for proponents of reef restoration as a central mechanism to restore oyster resources is not so much the demonstration of biological recruitment in the field as the social and political recruitment of citizens to support such efforts on a long-term basis. Demonstration of "success" in field programs, such as the recruitment event associated with reef construction and broodstock "seeding" in the Great Wicomico River in 1997, provide a vehicle to educate the public and foster vested interest groups. The target audience here is broad, as demonstrated by success to date in developing partnerships, which is illustrated by the following examples. Established environmental nonprofit groups, such as the Chesapeake Bay Foundation, use their considerable resources and infrastructure to support reef efforts on a regional basis. In stark contrast to the "not in my back yard" mentality associated with environmentally adverse programs, reefs are environmentally attractive structures that are desired "in my back yard." Consequently, local citizens groups sponsor reefs in their own "back yards" and school groups grow oysters to seed local reefs as part of the restoration effort. Currently lacking from this team is strong endorsement of both the commercial and recreational fishing communities in the bay. This is surprising, given the obvious long-term advantage to both, but probably reflects the immediacy of benefit that is required to attract these groups. Education is the avenue to forge this relationship, as demonstrated by the active support enjoyed by SAV restoration efforts from the fishing community. An integral part of this education must be the demonstration of the economic value of an ecological asset not just in terms of the commercial and recreational end product. It must be evident that that there is a cumulative positive impact of restored ecosystems in nutrient processing that is preferable to the current "single-payment option" exercised by some point-source nutrient abatement policies. The challenge remains to enjoin a broad citizen base in supporting ecological restoration on a broad base, understanding that they have vested interest as long-term investors in the watershed in which they communally reside with the Chesapeake Bay flora and fauna.

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PROGRESSION OF DISEASES CAUSED BY THE OYSTER PARASITES, *PERKINSUS MARINUS* AND *HAPLOSPORIDIUM NELSONI*, IN *CRASSOSTREA VIRGINICA* ON CONSTRUCTED INTERTIDAL REEFS

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ABSTRACT The progression of diseases caused by the oyster parasites *Perkinsus marinus* and *Haplosporidium nelsoni* were evaluated by periodic sampling (May 1994–December 1995) of eastern oysters *Crassostrea virginica* on an artificial reef located in the Piankatank River, Virginia. The infections observed were recorded as a function of: (1) prevalence and intensity; (2) oyster size and age; and (3) depth below mean low water at which the host oyster was found on the reef. Only a very small number of oysters were infected with the two species of pathogens on the oyster reef during the first 11 months of life. In the second year of oyster life, epizootiological patterns of disease development followed temperature and salinity trends. Oysters at residence depths ≤45 cm below mean low water exhibited significantly ($P < 0.0001$) lower prevalence and intensity of infections than oysters at depths ≥90 cm. In contrast, oysters at residence depths ≥90 cm had significantly higher growth rates ($P < 0.05$) than those at ≤45 cm. However, size differences were not significant ($P > 0.05$) at the end of the study. Results from this study may be used in managing oyster fisheries on natural or artificial reefs.

KEY WORDS: *Crassostrea virginica, Perkinsus marinus, Haplosporidium nelsoni*, artificial reefs, disease progression, growth

INTRODUCTION

Eastern oysters *Crassostrea virginica* were an economic and ecological resource in the Chesapeake Bay until the early 1900s (Hargis and Haven 1999). Years of overharvesting, diseases caused by the pathogens *Perkinsus marinus* and *Haplosporidium nelsoni*, environmental degradation, and poor resource management have led to a dramatic decline in oyster populations in the Chesapeake Bay (Andrews 1988, Haskin and Andrews 1988). Today, Virginia's oyster population is less than 1% of what it was just 35 years ago. (Wesson et al. 1999). Various efforts have been initiated to rejuvenate dwindling local oyster populations. These include spreading of hatchery-reared juvenile oysters on natural oyster beds in estuaries, spreading oyster shell in an attempt to increase hard substrate for settlement of oyster larvae, and construction of artificial reefs. Rejuvenation efforts, such as spreading thin layers of shell over coastal and estuarine bottom for larval attachment have had limited success. This may be due in part to the lack of three-dimensional complexity observed in natural intertidal communities. Little is known about the colonization and ecology of *C. virginica* on intertidal reefs. In addition, the advantages of oyster settlement on constructed reefs are not well understood. Therefore, this study was conducted to determine: (1) whether residence depth influenced the extent or intensity of disease infection; and (2) the size and age at which oysters became infected and the depths that resulted in significant infections. Observations from the present study are of interest to individuals responsible for constructing artificial reefs and individuals who must determine when to harvest oysters to avoid excess losses.

METHODS AND MATERIALS

Perkinsus marinus Infections

Oysters were assayed for the presence of *P. marinus* using the Ray's fluid thioglycollate medium technique (Ray 1954). Samples of gill and digestive gland were incubated in the medium. Perkins (unpublished data) determined that these organs can more frequently detect the presence of the parasite when infections are very light or light than when mantle or rectal samples are used. The

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intensity of infections was recorded using a modification of the Mackin scale (Mackin 1962) in which 0 = no infection, 1 = very light, 2 = light, 3 = light–moderate, 4 = moderate, 5 = moderate–heavy, and 6 = heavy.

Haplosporidium nelsoni Infections

Haplosporidium nelsoni was detected using histological, paraffin-embedded sections stained in hematoxylin and eosin. The scale of Burreson et al. (1988) was employed to record intensities of infections where 0 = no infection, 1 = cells were rare, 2 = fewer than two cells per field of view (40× objective), 3 = two–five cells per field of view, and 4 = more than five cells per field of view.

Oyster Sampling

From May 5, 1994 to December 14, 1995, the progression of diseases caused by the oyster parasites *P. marinus* and *H. nelsoni* were evaluated by periodic sampling of oysters that had naturally set on the artificial reef located in the Piankatank River, Virginia, in August 1993. The reef was constructed by the Virginia Marine Resource Commission using aged oyster shells. Details of reef construction are described elsewhere (Bartol and Mann 1997). Sampling of oysters was conducted once every 2 to 4 weeks during the study period. Oysters were obtained by hand or by using oyster tongs, depending on the depth. Six samples of 25 oysters each were obtained for each sample time at two locations on the reef. The base of the reef was 2–3 m below mean low water. Prevalence and intensity (weighted prevalence) of *P. marinus*, and *H. nelsoni* infections, oyster size and age, and depth below mean low water at which the host oyster was found on the reef were measured. The total number of reef oysters sampled was 3,908. With respect to depth, the data were analyzed according to the oyster's residence depth on the reef: ≤45 cm and ≥90 cm mean low water. The observations are expressed in terms of number of weeks after setting. Most of the set in 1993 occurred from August 5 to August 12. To facilitate the handling of the data, August 12 was selected as the date of set.

The progression of infections in the reef-set oysters were compared with the progression through a population of adult oysters. Uninfected, adult oysters (350) were obtained from the upper James River seed beds (Horsehead rock), a region known to have low *P. marinus* and *H. nelsoni* infections in oysters (Burreson and Ragone-Calvo 1996). These adult oysters were placed in plastic mesh bags and then were placed on the Piankatank River reef near the sample sites for the reef oysters; placement was June 16, 1994, the time of the third sampling of the reef oysters. The depth of placement was about midway between the top and bottom of the reef (ca. 100 cm below mean low water). At the time of placement, a sample of 25 oysters was analyzed for the presence of the two parasites, using techniques described previously. To confirm that the parasite detection methodology was being properly applied and to check for patchiness in distribution of the parasites, 350 James River oysters from the same population used on the Piankatank River reef were placed in plastic mesh bags in the York River behind the Virginia Institute of Marine Science (VIMS), an area in which both diseases are commonly present at high levels. Treatment of the 350 oysters held at VIMS was the same as described for the 350 adult oysters deployed in the Piankatank River. Each batch of 350 oysters was sampled (25 oysters per sampling time) simultaneously with the reef oysters until none remained. In the following spring (April 14, 1995), another 350 oysters from the same James River site were placed on the reef and 350 at VIMS as in the previous year and sampled until none remained.

Statistical Analyses

The effects of depth and sampling time (age) of oyster on disease susceptibility (prevalence and intensity of infection) to *P. marinus* and *H. nelsoni* were examined using logistic regression analysis (Agresti 1990). The differences in growth of oysters sampled at ≤45 cm and ≥90 cm depths during different sampling times was assessed using a two-way analysis of variance (ANOVA)

RESULTS

Temperature and Salinity

Temperatures and salinities during the study period showed typical seasonal patterns, higher temperatures during summer months and lower during winter months (Fig. 1). Salinity remained fairly constant during the study period. Temperature and salinity ranges during the study period were 2–30 °C and 10–20 ppt. The conversion between oyster age and sampling dates is presented in Table 1.

Perkinsus marinus Infections

No *P. marinus* or *H. nelsoni* cells were found in the 25 adult oysters sampled before the deployment of the oysters at Piankatank River and VIMS. *P. marinus* prevalence (Fig. 2a) and intensity (Fig. 2b) in oysters set on the Piankatank reef indicate that infections did not appear until 14 weeks into the study when the oysters were 1 year old (Table 1). For the next 44 weeks or until the oysters became 1 year and 10 months old, the number of infected oysters ranged between 15 and 35%; in the ensuing 2 months, the prevalence rose rapidly to 100%. Oysters then exhibited high prevalence (nearly 100%) until the end of the study, when the oysters were almost 2.5 years old. The infection intensities during the plateau phase remained mostly below very light until the end of the plateau (1 year, 10 months old), rose rapidly to moderate to moderate–heavy at 2+ years old, followed by a decline to light and light–moderate at the end of the study.

Perkinsus marinus prevalence was significantly higher ($P <$

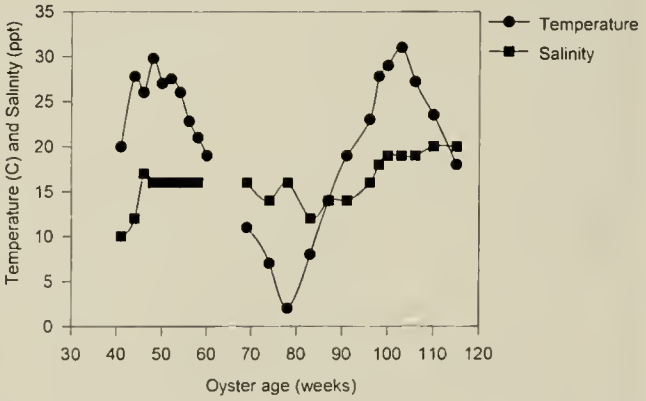

Figure 1. Temperatures and salinities at the reef site during the study as a function of oyster age. The conversion of oyster age to correspond to sampling dates is presented in Table 1. Discontinuities in the curves denote lack of data.

TABLE 1.

Time scale for sampling times used in the study. The oyster ages are estimated assuming a setting time of August 12, 1993. NA = Not available.

Sampling Date	Oyster Age (weeks)	Sampling Time Elapsed (weeks)	Temperature (°C)	Salinity (ppt)
May 5, 1994	38	0	NA	NA
May 26	41	3	20	10
June 16	44	6	27.8	12
June 30	46	8	26	17
July 15	48	10	29.8	16
July 28	50	12	27	16
August 12	52	14	27.5	16
August 26	54	16	26	16
September 8	56	18	22.8	16
September 23	58	20	21	16
October 5	60	22	19	NA
October 20	62	24	NA	NA
November 11	65	27	NA	NA
December 8	69	31	11	16
January 12, 1995	74	36	7	14
February 10	78	40	2	16
March 13	83	45	8	12
April 14	87	49	14	14
May 11	91	53	19	14
June 15	96	58	23	16
June 30	98	60	27.8	18
July 13	100	62	29	19
July 31	103	65	31	19
August 24	106	68	27.2	19
September 18	110	72	23.5	20
October 24	115	77	18	20
December 14	122	84	NA	NA

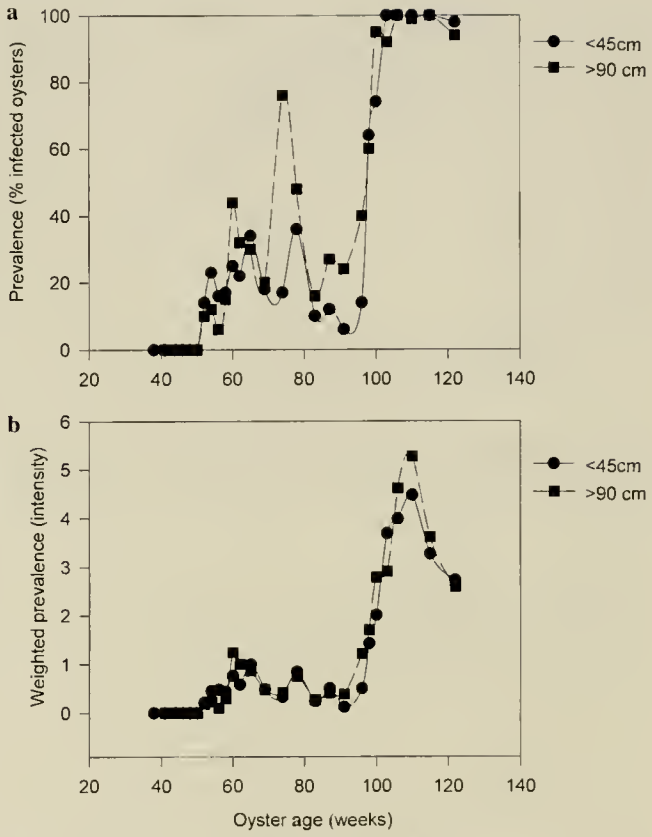

Figure 2. Prevalences (a) and intensities (weighted incidences) (b) of *Perkinsus marinus* infections in Piankatank River reef oysters that set in August 1993 are presented as a function of oyster age and depth of residence below mean low water (≤45 cm and ≥90 cm).

0.0001) in oysters collected from depths ≥90 cm compared to those from ≤45 cm (Fig. 2a). Prevalence significantly increased ($P < 0.0001$) in oysters from all depths with increasing age of oysters, indicating that continued exposure to *P. marinus* or increasing age of oysters results in increased infection. Similar results were observed when *P. marinus* infection was expressed as weighted prevalence. Although the difference in infection intensity was not as great as the prevalence, it was significantly higher at the greater depths ($P < 0.01$) and significantly increased in oysters from both depths with age ($P < 0.0001$).

Perkinsus marinus infection prevalence was the same in adult oysters held in plastic mesh bags at the York River and at the Piankatank River reef during 1994. In 1995, infection was expressed earlier at the Piankatank River and reached 100% 15 weeks before those held in the York River (Fig. 3a,b).

Haplosporidium nelsoni Infections

Prevalence of *H. nelsoni* was markedly different from that of *P. marinus* in reef oysters (Fig. 4a). With the exception of one lightly infected, 50-week-old oyster, the onset of *H. nelsoni* infections did not occur until the oysters were over 1.5 years old, as opposed to the appearance of *P. marinus* in 1-year-old oysters. Thereafter, the infection prevalence of *H. nelsoni* rose rapidly, reaching a maximum of 45% when the oysters were 21 months old. The infections then declined precipitously to almost 0% when the oysters were over 2 years old, followed by a slight increase, which remained below 10% for the final 4 months of the study. The intensities of infections peaked at the age of nearly 2 years, which was 1 month before the prevalence peak was reached, and declined almost as rapidly as did the prevalence (Fig. 4b).

Similar to *P. marinus* infections, oysters collected from ≥90 cm depth had a significantly higher prevalence and intensity of *H. nelsoni* infections compared to those from ≤45 cm depth ($P < 0.0001$) (Fig. 4a,b). In addition, *H. nelsoni* prevalence and intensity increased with increasing oyster age ($P < 0.0001$).

Haplosporidium nelsoni infections (Fig. 5a,b) were nearly nonexistent in the Piankatank River reef oysters during 1994; whereas, in the York River stock, infections were above a prevalence of 60% during the summer and fall of 1994.

Growth and Size

The sampling time and the residence depth of oysters significantly influenced the growth of oysters ($P < 0.001$) (Fig 6). Oysters at both depths grew with increasing age ($P < 0.001$) and sampling time (P 0.001). Oysters at depths ≥90 cm had significantly greater growth than oysters at depths ≤45 cm. Although the differences in size were pronounced during age 56–100 weeks, they were less pronounced during oyster ages 103–122 weeks, and insignificant ($P > 0.05$) at the end of the study. The rate of oyster growth, as estimated from size measurements, decreased 4 weeks

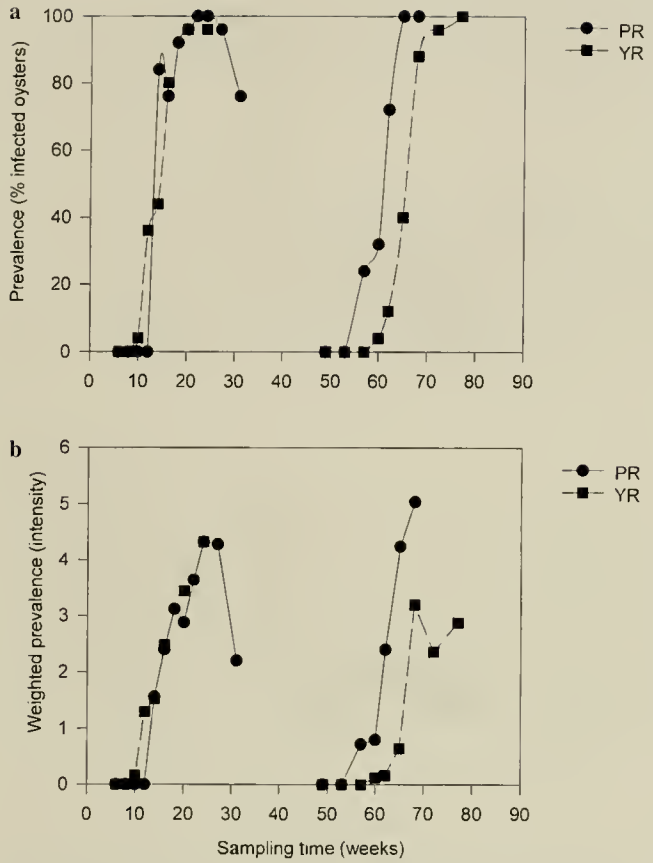

Figure 3. Prevalence (a) and intensity (weighted incidences) (b) of *Perkinsus marinus* for adult oysters imported from the upper James River and placed on the Piankatank River reef and in the York River behind the Virginia Institute of Marine Science. Oysters (350 at each site) were twice placed at the sites and assayed until the populations were depleted by sampling and natural mortalities. The disease organism data are expressed as a function of site and sampling time in the study.

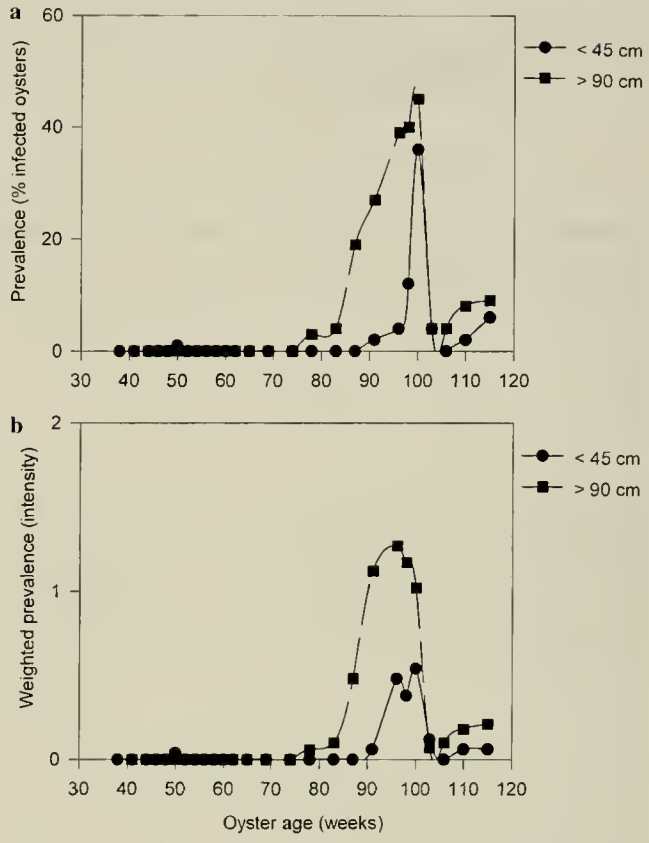

Figure 4. Prevalences (a) and intensities (weighted incidences) (b) of *Haplosporidium nelsoni* infections in Piankatank River reef oysters that set in August 1993 as a function of oyster age and depth of residence below mean low water (≤45 cm and ≥90 cm).

before the first *P. marinus* infections (10 weeks and 14 weeks into the study) (Fig. 6).

DISCUSSION

Interpretation of epizootiological data such as those generated in this study is confounded by many factors that dictate disease prevalence and intensity. These include temperature, salinity, water quality, density of oysters, patterns of water movement, oyster age and/or size, genetic strains, physiological condition, food availability (density and species composition of planktonic food organisms present), and numbers and levels of other parasitic species causing stress on the oysters. A further complication is the fact that the reservoir of *H. nelsoni* infective cells is unknown, and transmission of infections is not from oyster-to-oyster as with *P. marinus*. Despite these complicating factors, patterns of disease progression of both the diseases and oyster growth at different residence depths were apparent. The residence depth of the oysters relative to mean low water is of considerable interest, because the premise behind construction of artificial reefs is that the survival of oysters in the presence of *P. marinus* and *H. nelsoni* will be enhanced if they are grown in the more natural environment of an oyster shell reef off the bottom of the estuary. In fact, this study clearly indicates that residence depth of oysters significantly influences prevalence and intensity of *P. marinus* and *H. nelsoni* infections.

Oysters that are growing at ≤45 cm depth can be expected to have lower prevalences and intensities of infections of both pathogens compared to those living at ≥90 cm depth (Figs. 2a,b. and 4a,b). The prevalence and intensity of *P. marinus* in reef oysters from the current study, while agreeing with those of Mackin (1962), differ from studies by Quick and Mackin (1971) in the Atlantic and Gulf of Mexico coasts of Florida. Weighted incidences (intensities) in their study showed a decrease with increasing depth and no effect of depth on prevalences from intertidal to 3 m below mean low water. Similarly, Burrell et al. (1984) found higher prevalences and intensities of *P. marinus* in intertidal oysters than in subtidal oysters. Mackin (1962) speculated that lower infection prevalences and intensities in intertidal oysters may be because these oysters are not exposed to as many infective cells as subtidal oysters by virtue of the increased amount of time they are closed and not feeding.

Growth of oysters at both the depths (≤45 cm and ≥90 cm) decreased from age 74 weeks, and coincided with increased *P. marinus* incidence in oysters. The decrease in growth of oysters upon acquisition of *P. marinus* infections support the findings of Paynter and Burreson (1991) who observed a decrease in the growth rate of juvenile and adult oysters immediately after or just before infection. *Haplosporidium nelsoni* did not seem for more than 7 months after the change in growth rate, and it is concluded

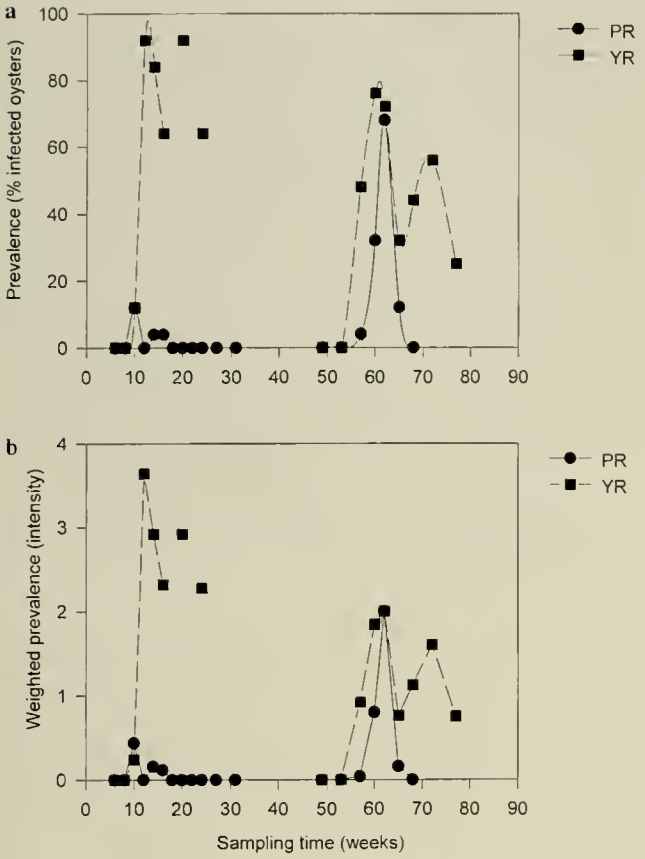

Figure 5. Prevalence (a) and intensity (weighted incidences) (b) of *Haplosporidium nelsoni* for adult oysters imported from the upper James River and placed on the Piankatank River reef and in the York River behind the Virginia Institute of Marine Science. Oysters (350 at each site) were twice placed at the sites and assayed until the populations were depleted by sampling and natural mortalities. The disease organism data are expressed as a function of site and sampling time in the study.

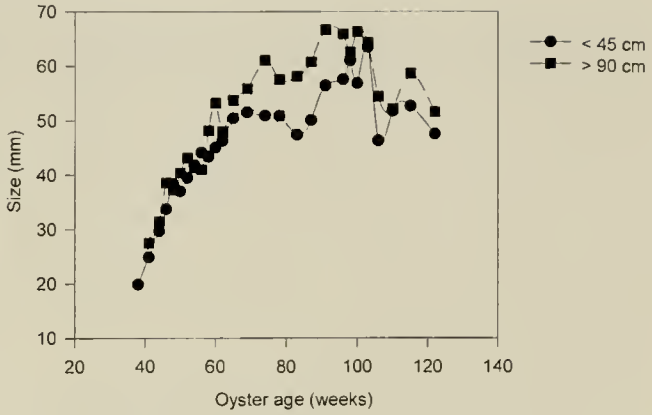

Figure 6. Piankatank River reef oysters that set in August 1993 and were sampled for the disease studies. Sizes are presented as a function of oyster ages and depth of residence below mean low water (≤45 cm and ≥90 cm).

that *P. marinus* was responsible for the decreased growth rate. The decline in oyster sizes after the age of 2 years (65 weeks into the study) is believed to be atributable to death of the larger oysters from infections of the two pathogens.

The higher growth of oysters residing at depths ≥90 cm than those at ≤45 cm is surprising. Given the higher infection prevalence and intensities of both *P. marinus* and *H. nelsoni* in oysters at depths ≥90 cm, we would expect that these oysters would grow less because of disease pressure. The biological, chemical, and physical processes associated with the bottom waters may influence the physiological and defense responses of organisms inhabiting these areas. Organic matter near the bottom of the reef close to the sediment has been speculated (Dahlback and Gunnarsson 1981) to have contributed to the increased growth in oysters from >90 cm. However, at the end of the study, the differences in sizes of oysters from the two depths were not significant.

The present dataset is unique, because it is the first time a population of naturally set oysters of known age has been assayed *in situ* for progression of infections by *P. marinus* and *H. nelsoni* over an extended period of time. Other epizootiological studies have involved placing naturally set or hatchery set oysters of known age in containers in an endemic area or placing adult oysters of unknown ages from nonendemic or marginally endemic areas into containers in an endemic area (Ray 1953, 1954, Mackin 1962, Paynter and Burreson 1991). Placement in containers provides a greater degree of experimental control, but artificial densities of oysters can lead to results different from those in naturally set populations, where distances vary from oyster to oyster.

The prevalence and intensities of infections of *P. marinus* in reef oysters generally followed the patterns dictated by temperature and salinity trends observed in earlier studies (e.g., Soniat 1985, Andrews 1988, Burreson 1991, Burreson and Ragone-Calvo 1996, Ray 1996). *P. marinus* infections in oysters rose in the spring, peaking in October and November, and declined in the winter months into the spring. Intensities of infections were more nearly reflective of previous reports in that the peaks for the two depths were in October and November and the minima in May of the following year (Fig. 2b). Therefore, assuming that temperature and salinity values approximate those of the study period and given the infection intensities and levels, oyster mortalities from *P. marinus* can be expected to begin 13 months after setting, rising most significantly 22 months after setting.

Considering the fact that the salinity values recorded during the first year after setting did not go below 10 ppt, and most of the time were ≥16 ppt, it is reasonable to assume that infections from these two diseases did not occur before May 5, 1994, when sampling began. This assumption is based on other studies that observed when a population becomes infected, the infections do not disappear (or decline) unless the salinity decreases below 10 ppt for an extended period (Andrews and Hewatt 1957, Ragone and Burreson 1993, Burreson and Ragone Calvo 1996).

Previous studies have noted that oysters are refractory to acquiring infections in the first year of life (Ray 1953, 1954) and become increasingly more susceptible into the second year, with significant prevalence, incidence, and mortality being observed then (Andrews and Hewitt 1957). That pattern was observed in the present study (Figs. 2a,b and 4a,b). As mentioned above, the complicating factor was the low level of infection pressure from *H. nelsoni* in the first year of life at the reef. *P. marinus* infections in oysters are dose-dependent (Mackin 1962, Chu and Volety 1997), and small oysters probably filter less volumes of water to acquire enough infective cells of the parasite to initiate an infection (Burreson 1991, Burreson and Ragone Calvo 1996). Results from the current study indicate that in the first 11 months of life, only a very small number of oysters on the oyster reef become infected with

the two species of pathogens. The question that cannot be answered is whether the primary factor in encouraging or permitting a rapid increase in prevalence was temperature, oyster age, or salinity. The best answer probably is that all three played interactive roles.

Data for adult oysters imported from the upper James River seed beds, which represent a disease-susceptible population, confirmed that the two pathogens were present in the study area and in the neighboring area of the lower York River (Figs. 3a,b and 5a,b). They were used primarily to indicate presence or absence of *H. nelsoni*, because it is known to fluctuate greatly, some years being nearly absent from the lower York River region. (Andrews 1988, Haskin and Andrews 1988).

It is interesting to note that *P. marinus* infection prevalence was the same in oysters held in the York River and at the Piankatank River reef during 1994, but in 1995 was expressed earlier in the Piankatank River and prevalence reached 100%, 15 weeks before those held in the York River. One would have expected that the oysters held in the York River would have shown a higher prevalence, because salinities were about 5 ppt above those of the Piankatank River and thus would have presented more favorable salinities for expression of *P. marinus*. On the other hand, in 1994, *H. nelsoni* infections were nearly nonexistent in the Piankatank River reef oysters; whereas, in the York River stock, infections were above a prevalence of 60% during the summer and fall of 1994 (Fig. 5a). These observations reaffirm the necessity of having a stock of susceptible, adult oysters present in a study where juvenile and young oysters are being observed.

The reef oysters ≥2 years old can reasonably be compared to the imported oysters in terms of response to the diseases. Although *P. marinus* prevalences and intensities of infections were similar in the two groups of oysters at the reef, *H. nelsoni* data suggest that the reef-set oysters were more resistant to those infections. Imported oysters at the reef reached a peak of 68% infection (Fig. 5a); whereas, the reef oysters peaked at 36 and 45%, depending upon the depth of residence (Fig. 4a). Likewise, the intensities of *H. nelsoni* infections had a mean level of 2 in imported oysters as opposed to 1.3 in the reef oysters. Therefore, some advantage seems to have been obtained for the reef-set oysters, if we can neglect minor age differences.

The data for *H. nelsoni* was somewhat surprising, because only one oyster was found to be infected in the first year of life (Fig. 4a), and the population did not otherwise begin to show infections until the oysters were over 1.5 years old. It is possible that this lag can be attributed to: (1) the oysters being young and, thus, less susceptible, as has been reported from other studies; and (2) the fact that even the susceptible, imported adult oysters did not acquire very many infections at the Piankatank River reef (Fig. 5a) in the first year of life. It was clear that *H. nelsoni* was present in strength in the nearby York River (Fig. 5a) but not in the reef area, thus illustrating the patchiness in distribution of the reservoir of infective cells, at least in that part of the Chesapeake Bay. The decline in prevalence and intensity of *H. nelsoni* in reef oysters (Fig. 5a,b) was more precipitous that has been previously reported using imported susceptible adult oysters (Haskin and Andrews 1988). In 1995, a different picture of *H. nelsoni* infection distributions in the imported, adult oysters was observed (Figs. 5a,b). The prevalences and intensities were quite similar at the two stations, with the infections appearing earlier at the York River station and lasting longer in the population. Nine more weeks of data were obtained from the York River stock, because the stock at the reef was depleted by mortalities earlier, probably caused by *P. marinus* infections (Fig. 5a,b).

Given the decreased susceptibility of oysters to *P. marinus* and *H. nelsoni* at residence depth of ≤45 cm compared with oysters at ≥90 cm, it seems that piling of shells and constructing artificial reefs is a better strategy for rejuvenating oyster stocks than spreading thin layers of oyster shells on the bottom in estuarine and coastal areas. Future studies should investigate the survival differences in oysters at different depths and the factors responsible for differences in growth rates of oysters at different depths.

ACKNOWLEDGMENTS

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PREVALENCE OF *PERKINSUS* SPP. IN CHESAPEAKE BAY SOFT-SHELL CLAMS, *MYA ARENARIA* LINNAEUS, 1758 DURING 1990–1998

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ABSTRACT Prevalence and intensity of *Perkinsus* spp. infections were determined in soft-shell clams *Mya arenaria* during 1990 to 1998 based upon incubation of rectal tissues in Ray's fluid thioglycollate medium. During the study, soft-shell clams were collected from 18 sites in the upper Chesapeake Bay in Maryland. Enlarged hypnospores were found in ~7% (114/1,705) of the soft-shell clams. Peak prevalences occurred in the fall of 1992 with ~53% (16/30) at Piney Point and 50% (15/30) at Eastern Neck, and in August 1995 with ~64% (18/28) and ~37% (11/30) at Cedar Point and Piney Point, respectively. This investigation provides evidence that *Perkinsus* spp. infections in soft-shell clams are more common than previously thought.

KEY WORDS: soft-shell clam, *Perkinsus* spp., Chesapeake Bay, Maryland, infection, intensity

INTRODUCTION

Protozoa of the genus *Perkinsus* have been associated with significant losses of feral and cultured species of bivalve mollusks worldwide. Previous reports of *Perkinsus* sp. infections in soft-shell clams *Mya arenaria* of the Chesapeake Bay have been uncommon (Andrews 1954), and its geographic distribution remains to be determined. Recently, McLaughlin and Faisal (1998a) reported the presence of *Perkinsus* spp. in Maryland soft-shell clams and described the associated histopathological alterations. Most of the infections observed were light in intensity, limited to the gills and palps, and evoked cellular host responses including encapsulation of invading parasites. As infection intensity increased, the parasite was found in nearly all tissues of the soft-shell clam, sometimes causing adverse host effects (McLaughlin and Faisal 1998a, 1999). Interestingly, two species of *Perkinsus* were recently isolated from hemolymph and gills of soft-shell clams collected from the Chesapeake Bay and propagated *in vitro* (McLaughlin and Faisal 1998b). Morphology, life cycle, and molecular characterization studies showed similarities between the soft-shell clam hemolymph isolate and *P. marinus*, and provided evidence that the gill isolate was an undescribed *Perkinsus* sp. (McLaughlin and Faisal 1998a,b, Kotob et al. 1999a,b).

In the assay routinely used for the detection and quantitation of *Perkinsus* spp. cells in bivalves, host tissues are incubated in Ray's fluid thioglycollate medium (RFTM) and enlarged hypnospores then stained with Lugol's iodine (Ray 1952). In soft-shell clams, the use of rectal tissue in thioglycollate assays was found to be effective for diagnosing advanced *Perkinsus* spp. infections (McLaughlin and Faisal 1999). A positive result implies that the infection has progressed from the early encapsulation stage within gill tissues to a more systemic infection spreading into various tissues of the infected clam. In this paper, we report infection prevalences and intensities of *Perkinsus* spp. in *Mya arenaria* collected from 18 sites in the Chesapeake Bay during 1990 to

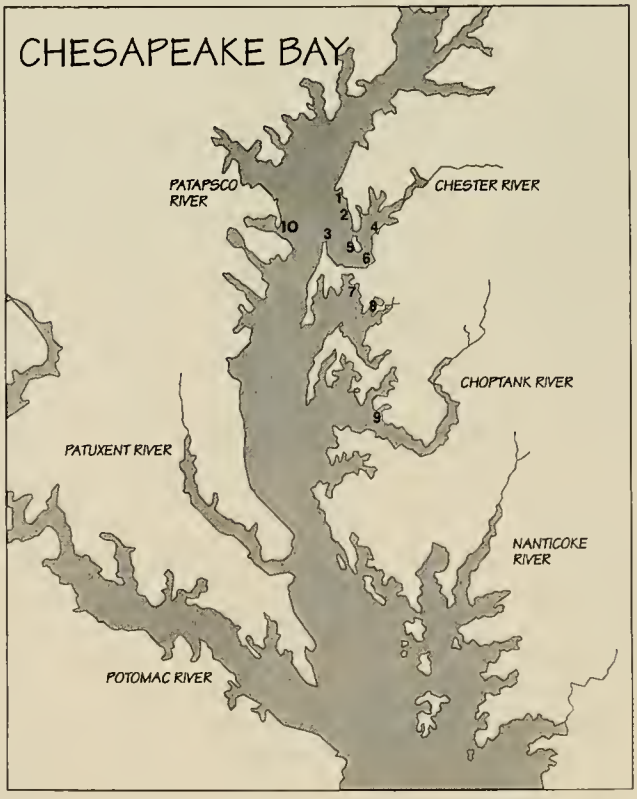

Figure. 1. Sampling sites in the upper Chesapeake Bay (1 = Swan Point; 2 = Eastern Neck; 3 = Love Point; 4 = Piney Point; 5 = Eastern Neck Island; 6= Cedar Point; 7 = Cabin Creek; 8 = Wye River; 9 = Howell's Point; 10 = Gibson Island) with *Perkinsus*-positive soft-shell clams *Mya arenaria* based upon incubation of rectal tissues in Ray's fluid thioglycollate medium (Ray 1952). Scale: 1 cm = ~6.9 miles (~11.04 km)

TABLE 1.

Prevalence (percent infected) and intensity (weighted prevalence) (Mackin 1962) of *Perkinsus* spp. in softshell clams *Mya arenaria* from sites in the upper Chesapeake Bay (n = 30).

Location	Date	Temperature (° C)	Salinity (ppt)	Percent Infected	Weighted Prevalence
Swan Point	December 1990	9.0	7.5	7	0.07
Swan Point	March 1991	8.0	5.0	0	0.00
Swan Point	July 1991	27.5	10.5	0	0.00
Howell's Point	August 1991	20.0	9.2	10	0.27
Wye River	August 1991	25.4	12.5	3	0.03
Swan Point	October 1991	20.0	12.5	23	0.33
Swan Point	January 1992	5.0	14.5	3	0.03
Little Choptank	March 1992	—	—	0	0.00
Love Point	April 1992	7.7	11.5	3	0.03
Swan Point	April 1992	5.0	10.0	0	0.00
Piney Point	April 1992	9.9	9.3	0	0.00
Bishop Head Point	April 1992	—	—	0	0.00
Sandy Point	April 1992	12.8	3.8	0	0.00
Gibson Island[1]	April 1992	8.0	12.0	0	0.00
Cabin Creek	June 1992	24.9	13.1	7	0.06
Swan Point	July 1992	3.6	11.0	0	0.00
Love Point	July 1992	25.8	10.8	0	0.00
Sandy Point	July 1992	23.8	10.0	0	0.00
Wye River	July 1992	23.8	13.0	0	0.00
Swan Point	September 1992	0	12.0	7	0.10
Piney Point	September 1992	22.6	5.0	53	1.17
Gibson Island	October 1992	15.9	16.3	26	0.40
Eastern Neck	October 1992	13.6	14.0	50	1.50
Swan Point	February 1993	3.0	7.0	0	0.00
Nichols Point	June 1993	20.4	4.0	0	0.00
Piney Point	June 1993	19.1	4.2	0	0.00
Cedar Point[2]	June 1993	17.0	4.2	0	0.00
Swan Point	August 1993	25.1	10.0	17	0.02
Piney Point	August 1993	25.0	9.8	10	0.30
Eastern Neck	August 1993	25.1	10.0	10	0.20
Love Point	August 1993	25.3	11.0	0	0.00
Gibson Island	August 1993	26.0	8.2	0	0.00
Rock Point	August 1993	—	—	0	0.00
Swan Point	October 1993	13.7	14.0	3	0.03
Swan Point	March 1994	4.4	5.0	0	0.00
Swan Point	June 1994	26.6	3.5	0	0.00
Love Point	June 1994	22.0	8.0	0	0.00
Sandy Point	June 1994	25.0	4.9	0	0.00
Eastern Neck Island	June 1994	25.0	5.5	0	0.00
Pier 1	June 1994	26.2	5.0	0	0.00
Love Point	July 1994	26.6	5.0	0	0.00
Swan Point	July 1994	26.6	3.5	0	0.00
Eastern Neck	August 1994	25.2	6.5	0	0.00
Swan Point	August 1994	25.6	7.1	0	0.00
Huntingfield	September 1994	22.2	10.0	0	0.00
Swan Point	September 1994	22.2	9.0	0	0.00
Swan Creek	September 1994	22.2	8.0	0	0.00
Swan Point	February 1995	2.2	6.0	10	0.10
Piney Point	February 1995	3.1	6.0	3	0.03
Eastern Neck Island	February 1995	3.1	6.0	3	0.03
Swan Point	July 1995	24.2	6.0	0	0.00
Eastern Neck Island	July 1995	25.0	10.0	0	0.00
Cedar Point[1]	August 1995	23.5	14.0	64	1.57
Piney Point	August 1995	23.6	13.5	37	1.33
Swan Point	August 1995	23.0	12.5	13	0.20
Swan Point	October 1996	15.6	2.2	13	0.20
Swan Point	July 1998	25.6	4.0	7	0.10

[1] n = 28.

[2] n = 29.

Prevalence and intensity were based upon incubation of rectal tissues in Ray's fluid thioglycollate medium (Ray 1952).

1998. Prevalences and intensities were determined using rectal tissues in thioglycollate assays.

MATERIALS AND METHODS

Clam Collections

More than 1,700 soft-shell clams were collected in 57 samples (~30 clams/sample) from 18 sites in the Chesapeake Bay (Fig. 1) from 1990 to 1998 by hydraulic escalator dredge. Clams from each sampling site were held on ice and transported immediately to the wet lab facility at the Cooperative Oxford Laboratory (COL), Oxford, Maryland. Clams were held in 76-L glass aquaria supplied with Tred Avon River water or artificial seawater at temperatures between 8 and 20 °C until processed (1–2 days). Salinities were adjusted to approximate those of the collection sites (5–16 ppt).

Ray's Fluid Thioglycollate Assays

Pieces of rectum were excised from each clam and incubated in RFTM (Ray 1952) for 5–7 days. Incubated tissues were subsequently macerated on glass slides, stained with Lugol's iodine, and examined for spherical blue-black bodies characteristic of *Perkinsus* spp. (Ray 1952). Infection intensity was estimated using a semiquantitative scale from 0 (negative) to 7 (extremely heavy infection), modified from Ray (1954) and Mackin (1962). Weighted prevalences were determined by adding the individual assigned intensities and dividing by the number of clams sampled (Mackin 1962).

RESULTS

Perkinsus spp. infections were found in ~7% (114/1,705) of soft-shell clams examined. Infected clams were found at 10 of the 18 sites surveyed (Fig. 1). Temperatures and salinities at sites positive for *Perkinsus* spp. ranged from 2.2 to 25.4 ° C and from 2.2 to 16.3 ppt (Table 1). Peak prevalences of soft-shell clam *Perkinsus* spp. usually occurred in the late summer and fall when salinities and temperatures were highest. A peak in prevalence occurred in the fall of 1992 with ~53% (16/30) at Piney Point and 50% (15/30) at Eastern Neck (Table 1). Prevalence also peaked in August 1995 with ~64% (18/28) and ~37% (11/30) at Cedar Point and Piney Point, respectively. As shown in Table 2, intensities of infections in the rectal tissues ranged from extremely light (stage 1) to heavy (stage 5). Extreme cases (stages 6 and 7) were observed in less than 1% (9/1705) of the soft-shell clams examined. The maximum weighted prevalences observed were 1.17 at Piney Point in September 1992 and 1.57 at Cedar Point in August 1995.

DISCUSSION

Previous reports of *Perkinsus* spp. infections in soft-shell clams are rare. In Virginia, *Perkinsus* sp. was reported to occur infrequently in soft-shell clams and the low infection intensities observed were not associated with histopathological lesions or mortalities (Andrews 1954). Similarly, histological examination of over 3,500 soft-shell clams collected from 20 sites in Maryland during 1969 to 1989 revealed only occasional occurrences of *Perkinsus* spp. (Sara V. Otto, Maryland Department of Natural Re-

TABLE 2.

Infection intensities of *Perkinsus* spp. in softshell clams, *Mya arenaria*, from sites in the upper Chesapeake Bay based upon incubation of rectal tissues in Ray's fluid thioglycollate medium (Ray 1952).

Site (n = 30)	Date (month & year)	Intensity of Infection (Stages 1–7) 1 # (~%)	2 # (~%)	3 # (~%)	4 # (~%)	5 # (~%)	6 # (~%)	7 # (~%)	Total # (~%)
Swan Point	December 1990	2 (7)							2 (7)
Howell's Point	August 1991	1 (3)	1 (3)		1 (3)				3 (10)
Wye River	August 1991	1 (3)							1 (3)
Swan Point	October 1991	4 (13)	1 (3)		1 (3)			1 (3)	7 (23)
Swan Point	January 1992	1 (3)							1 (3)
Love Point	April 1992	1 (3)							1 (3)
Cabin Creek	June 1992	2 (7)							2 (7)
Swan Point	September 1992	1 (3)	1 (3)						2 (7)
Piney Point	September 1992	8 (27)	3 (10)	3 (10)		1 (3)		1 (3)	16 (53)
Gibson Island	October 1992	6 (20)	1 (3)		1 (3)				8 (27)
Eastern Neck	October 1992	6 (20)	2 (7)	1 (3)	1 (3)	2 (7)	2 (7)	1 (3)	15 (50)
Eastern Neck	August 1993		3 (10)						3 (10)
Swan Point	August 1993	4 (13)	1 (3)						5 (17)
Piney Point	August 1993	1 (3)	1 (3)					1 (3)	3 (10)
Swan Point	October 1993	1 (3)							1 (3)
Swan Point	February 1995	3 (10)							3 (10)
Piney Point	February 1995	1 (3)							1 (3)
Eastern Neck Island	February 1995	1 (3)							1 (3)
Cedar Point[1]	August 1995	5 (18)	5 (18)	3 (11)	2 (7)	3 (11)			18 (64)
Piney Point	August 1995	6 (20)	3 (10)	1 (3)			1 (3)		11 (37)
Swan Point	August 1995	2 (7)	2 (7)						4 (13)
Swan Point	October 1996	1 (3)			1 (3)			2 (7)	4 (13)
Swan Point	July 1998	2 (7)							2 (7)

[1] n = 28.

Stage 1 = extremely light, 2 = very light, 3 = light, 4 = moderate, 5 = heavy, 6 = very heavy, 7 = extremely heavy.

sources, Oxford, Maryland, pers. comm.). McLaughlin and Faisal (1999) demonstrated that positive thioglycollate assays using rectal tissue denote advanced, generalized infections of *Perkinsus* spp. in soft-shell clams. This observation was verified in histologic preparations. Hence, the findings from our present study suggest that *Perkinsus* spp. infections may be increasing in soft-shell clams of the Chesapeake Bay. Indeed, soft-shell clams have been shown to host more than one species of *Perkinsus*, one closely related to *P. marinus* and the other an undescribed *Perkinsus* sp. (McLaughlin and Faisal 1998a,b; Kotob et al. 1999a,b). The lack of an effective tool for distinguishing between species of *Perkinsus* within a host limits our ability to attribute the increased prevalence to one or both soft-shell clam *Perkinsus* species.

This apparent elevation in *Perkinsus* spp. infections in soft-shell clams of the Chesapeake Bay parallels increased prevalences and range extensions of *P. marinus* infections in the eastern oyster. Increased occurrences of the oyster parasite were attributed to high salinities from successive droughts during the 1980s, concurrent mild winter temperatures, and movement of infected oysters (Burreson and Calvo 1996). Range extensions of *P. marinus* parasites were further hypothesized to be associated with genetic changes in the host or parasite and/or environmental parameters (Ford 1996). Coincidental increases in soft-shell clam *Perkinsus* spp. in the Chesapeake Bay may be attributable to similar factors. For example, the extension of oyster *P. marinus* into all productive oyster grounds in the Chesapeake Bay in the late 1980s and early 1990s (Burreson and Calvo 1996) coincided with the increased occurrence of soft-shell clam *Perkinsus* spp. observed in this study. Indeed, *P. marinus* was first reported in oysters in Swan Point, Chester River, in 1987 (Burreson and Calvo 1996) less than 3 years before *Perkinsus* spp. infections were observed in soft-shell clams from the same site. Similarly, reduced infection levels of *P. marinus* in Chesapeake Bay oysters during 1994 (Burreson and Calvo 1996) were concurrent with reduced soft-shell clam *Perkinsus* spp. prevalences in the same year.

The high prevalences of soft-shell clam *Perkinsus* spp. at some sites in Maryland during 1992 coincided with the lowest recorded catch of soft-shell clams since 1962 (Connie Lewis, Maryland Department of Natural Resources, Annapolis, Maryland, pers. comm.). The continued low harvests of soft-shell clams in the Chesapeake Bay suggest further investigations on impacts of the parasite to *M. arenaria* fisheries are warranted.

ACKNOWLEDGMENTS

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SUMMER MORTALITY OF PACIFIC OYSTERS, *CRASSOSTREA GIGAS* (THUNBERG): INITIAL FINDINGS ON MULTIPLE ENVIRONMENTAL STRESSORS IN PUGET SOUND, WASHINGTON, 1998

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ABSTRACT A study was begun in late 1997 in Puget Sound, Washington, and Tomalas Bay, California, to characterize more precisely the summer mortality of the Pacific oyster (*Crassostrea gigas*) in a variety of culture conditions and locations and to describe definitively the relationship of summer mortality to infectious diseases. Water quality and seasonal factors also were identified. A field component investigated the oysters' thermal stress response and assessed induced thermal tolerance as a means to reduce mortalities. In addition, management practices for commercial cultivation were evaluated as measures to reduce the frequency and extent of oyster losses. Our evaluation of the 1998 data from the summer mortality project supports earlier reports on the rate and timing of mortality events. There were differences in the mortality rates among the varieties of oysters tested, with triploid oysters having consistently higher mortality rates than diploid oysters planted in comparable plots. Trends in mortalities were toward higher rates at or immediately after neap tides when dissolved oxygen was lowest and during periods of elevated air and water temperatures. Relative densities of the phytoplankton *Gymnodinium splendens, Ceratium* spp., and *Psuedo-nitzschia* spp. were higher during the late summer; dissolved oxygen concentrations were correspondingly low, and oyster mortalities were high during this same period. It is likely that Pacific oysters at the study sites experienced varying degrees of chronic stress attributable to multiple environmental factors. Evaluations of effects of those stressors and development of oyster health management strategies are continuing.

KEY WORDS: Pacific oyster, *Crassostrea gigas*, disease, mortality, environment

INTRODUCTION

Pacific oyster *Crassostrea gigas* (Thunberg) production on the U.S. West Coast has not experienced the catastrophic losses from disease plaguing the East Coast; however, mass mortalities occur periodically and continue to threaten commercial production. The syndrome known as summer mortality has been known on the Pacific Coast for at least 40 years. Sharp increases in mortality from June to September are the classic example of summer mortality. The magnitude of the losses has been estimated as up to 50% of the harvestable crop in a given summer, but losses are highly variable by specific location and year. Reduction of these high losses is an integral part of a larger Pacific oyster health management program, being carried out by West Coast research organizations and shellfish farmers, to increase production and sales of seed and edible Pacific oysters.

Background

As early as the 1940s, serious losses of Pacific oysters were reported from Japanese culture locations (Koganezawa 1974). Mortalities occurred during the summer months, and at times exceeded 60%. Although various age groups were affected during an episode, the more severe losses occurred in the older and larger oysters. On the west coast of North America, oyster mortalities have been reported since the late 1950s from Washington, California, and British Columbia (Glude 1975). Again, older oysters seemed to be more susceptible, but the timing and degree of loss was variable. The most severe episodes occurred in shallow, nutrient-rich embayments in late summer when seasonal temperatures were highest.

Japanese researchers advanced a theory of metabolic imbalance related to accelerated reproductive maturation (Imai et al. 1965, Tamate et al. 1965). They concluded that oyster mortalities were related to reproductive maturation and environmental conditions present in growing areas before the end of July. The thought was that mass mortalities in Matsushima Bay associated with high temperatures and nutrient-rich waters led to accelerated reproductive development.

In Washington state, a similar metabolic imbalance was proposed, although regional water temperatures are generally lower than those in the Japanese growing areas (Perdue 1983, Perdue et al. 1981). Scholz et al. (1973) believed mortality was associated with gonad resorption. These studies also noted a high variability in mortality rates between growing areas in close proximity.

Studies to produce resistant oysters and genetically altered broodstocks were conducted by the University of Washington in the 1970s and 1980s. Selective breeding experiments showed that survival could be increased significantly, but it was accompanied by a severe decline in production yields (Beattie 1984). Surviving oysters were smaller, slower growing, and thinner than susceptible oysters and had little commercial use. The subsequent development of triploid Pacific oysters, which have poorly developed gonads, offered a promising option to reduction of summer kills (Allen et al. 1989).

The role of infectious agents in summer mortalities of Pacific oysters and other *Crassostrea* species has been studied extensively in Asia, the United States, and Europe (Sindermann 1990, Elston 1993). Early studies by Japanese investigators discounted the role of a bacterial disease now known as nocardiosis, because a correlation with infection and mortality could not be established (Numachi and Oizumi 1965). However, nocardiosis has recently been associated with some episodes of summer kill (Elston et al. 1987, Friedman 1990, Friedman et al. 1991). A herpes-like virus infection has been seen in larvae and seed oysters of Pacific oyster from hatcheries on the Atlantic coast of France (Nicolas et al. 1992, Renault et al. 1994, Renault IFREMER, pers. comm. 1997). These authors reported summer mortalities of 80–90% in infected seed. Similar herpes-like infections have not been observed in U.S. West Coast Pacific oysters.

PURPOSE AND OBJECTIVES

In late 1997, the Sea Grant Oyster Disease Research Program funded a multiyear study to further investigate summer mortality in Pacific oysters. This project was designed to test hypotheses relating to mortalities of Pacific oysters on the U.S. West Coast and to recommend measures to reduce those mortalities. The specific objectives were the following.

1. Perform studies that will provide health and disease information for Pacific oysters in areas experiencing high frequencies of summer mortality.
2. Carry out a comprehensive survey of environmental conditions occurring in the affected areas.
3. Evaluate in field trials the influences of environmental factors on stress proteins, morbidity, and mortality of diploid and triploid Pacific oysters, under a variety of culture conditions.
4. Identify and test short-term mortality reduction options for shellfish farmers and resource managers; and make recommendations for longer term study.
5. Compile information on Pacific oyster mortality from shellfish farmers and researchers on the U.S. and Canadian West Coast.

This report describes the first-year activities and findings of that study. Project activities reviewed in this report include:

1. experimental design and setup of study sites and treatment groups in Puget Sound, Washington;
2. monitoring of mortality, condition indices, growth, and associated fauna and flora; and
3. monitoring of water quality parameters and phytoplankton.

Related activities during the same period, not reported here included:

1. compiling information on summer mortality events from Tomales Bay, California, and other locations; and
2. sampling for stress protein, conducting histopathology analyses, and assessing pathology associated with mortality.

METHODS AND MATERIALS

Site Selection

The original project design called for field sampling and screening stations in two locations in south Puget Sound (Mud Bay and Totten Inlet) and one in north Puget Sound (Sequim Bay). The Mud Bay and Sequim Bay sites were believed to have a high incidence of summer mortality; whereas, the Totten Inlet site was selected as a low mortality control. Each site was dedicated for the duration of the project on private oyster grounds owned by Taylor Shellfish Farms and the Jamestown S'Klallam Tribe.

Preliminary siting meetings were held with shellfish farmers and researchers in February 1998. As a result of these meetings, the decision was made that the project would benefit from two additional Puget Sound sites. One site was to be near the community of Allyn, in North Bay, south Puget Sound. This site was being intensively monitored for toxic phytoplankton and other marine algae, had experienced consistent annual mortalities and, in 1997, had very high levels of both *Vibrio parahaemolyticus* and paralytic shellfish toxicity (PSP). The second site was in Eld Inlet and located to continue observations on Pacific oysters imported in 1994 from Tasmania. During a 1997 to 1998 study, these oysters were found to have both lower mortality and higher growth rates than a comparable population of native Pacific oysters (Kittel 1998). Finally, late in the summer of 1998, a sixth site was added in Tomales Bay, California. Sampling of this site was directed at an histopathological analysis of seed mortality during the September to October period. No environmental or water quality data were gathered. The locations of each of the five Puget Sound sites are shown in Figure 1.

Site Design and Setup

The experimental design called for a combination of shellfish health, environmental, and general biological monitoring at each study site. In conjunction with pathogen screening, we monitored water quality, stress proteins, oyster size, condition, mortalities, associated fauna and flora, and other relevant features. Experiments were designed to challenge, under commercial conditions, seed, yearling, and market-ready oysters held on the bottom and in culture bags placed on the bottom and on racks. All plots were located in or immediately adjacent to commercial grounds.

All experimental sites in Puget Sound were established between mid-April and late May 1998. Approximate bottom elevations mean lower low water (MLLW) were: Sequim Bay 0.0 m (0 ft), Totten Inlet 0.9 m (3 ft), Mud Bay 0.3 m (1 ft), Allyn 0.3 m (1 ft), and Eld Inlet 0.0 m (0 ft). Nearly 26,000 diploid and triploid oysters of varying sizes were transplanted from existing farm beds and nursery facilities. An additional 2,000 oysters were stockpiled to provide replacement animals and specimens for histopathology and stress protein sampling. The make-up and density of oysters in the culture treatments at each site are shown in Table 1.

Instrumentation was also deployed for continuous or repeated

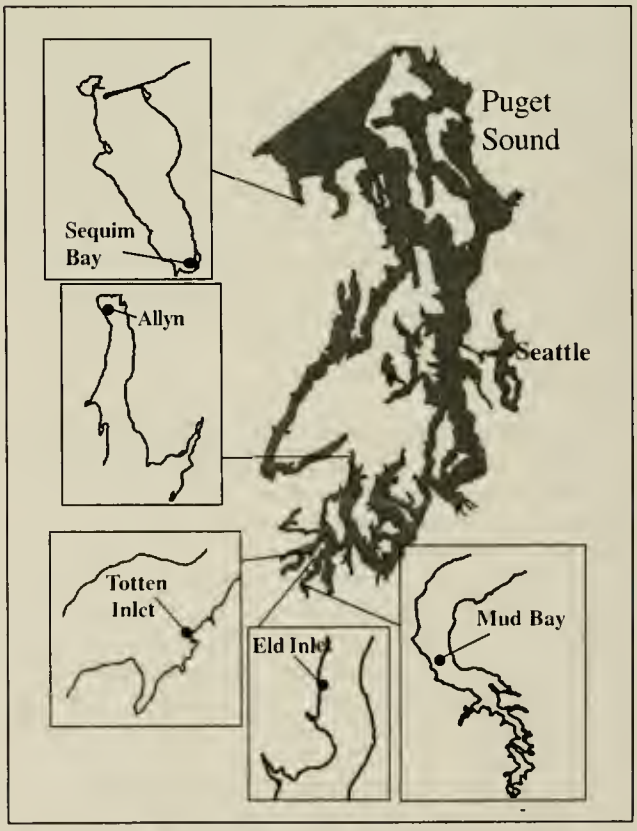

Figure. 1. Study sites Pacific oyster summer mortality project 1998–1999, Puget Sound, Washington. Lines and filled circles in the inset maps point to the 1998 summer mortality sampling sites.

TABLE 1.

Oyster type, treatment, culture type, sample unit, and number of oysters used at experimental oyster summer mortality study sites in Puget Sound, summer–fall 1998.

Oyster Type	Treatment	Culture Type[1]	Density	Unit	No. Units	Number Oysters			
						Allyn	Mud Bay	Sequim Bay	Totten Inlet
Diploid	Seed	Singles	50	/bag	3–6	300	0	300	150
			125	/bag	3–6	750	0	750	375
			200	/bag	3–6	1,200	0	1,200	600
Totals						**2,250**	**0**	**2,250**	**1,125**
	Seed	Cultched	100	/sq m	3.2	0	320	320	0
			300	/sq m	3.2	0	960	960	0
			650	/sq m	3.2	0	2,080	2,080	0
Totals						**0**	**3,360**	**3,360**	**0**
	Yearling	Cultched	80	/sq m	1.6–3.2	256	256	0	128
			300	/sq m	1.6–3.2	960	960	0	480
Totals						**1,216**	**1,216**	**0**	**608**
	Market-ready	Cultched	80	/sq m	1.6–3.2	256	256	256	128
			300	/sq m	1.6–3.2	960	960	960	480
Totals						**1,216**	**1,216**	**1,216**	**608**
	Market-ready	Singles	25	/bag	3–6	150	0	150	75
			75	/bag	3–6	450	0	450	225
			50	/bag	5	0	250	0	0
			40	/bag	15	0	600	0	0
Totals						**600**	**850**	**600**	**300**
Tasmanian	Market-Ready	Singles	50	/bag	5	0	250	0	0
			50	/bag	15	0	750	0	0
Totals						0	1,000	0	0
Total Diploid						**5,282**	**7,642**	**7,426**	**2,641**
Triploid	Yearling	Singles	80	/sq m	1.6	0	0	0	128
			300	/sq m	1.6	0	0	0	480
Totals						**0**	**0**	**0**	**608**
	Yearling	Singles	150	/bag	2	0	0	0	300
	Market-ready	Singles	80	/sq m	1.6–3.2	256	256	0	128
			300	/sq m	1.6–3.2	960	960	0	480
Totals						**1,216**	**1,216**	**0**	**608**
Total Triploid						**1,216**	**1,216**	**0**	**1,516**
Total Diploid and Triploid						**6,498**	**8,858**	**7,426**	**4,157**

[1] Cultched seed: 2–15 mm diameter seed attached to shell, 4–8 seed per shell; single seed: 15–30-mm-diameter singles; yearling: 1–2 years old and 80–100 mm-long singles; market ready: 2–3 years old 100–150-mm long singles or in clusters. These oysters were spread evenly on the bottom or placed in 1.0 × 0.5 = m 10-mm plastic-mesh growout bags.

monitoring of key water quality parameters. Onset© miniature data loggers were placed in waterproof cases and installed on the bottom at each study site to sample ambient water and air temperatures. We also assembled and deployed similar Onset© data loggers fitted with dissolved oxygen (DO) probes and operational amplifiers for long-term, continuous monitoring of DO.

Site Surveys and Monitoring

Field Sampling and Mortality Monitoring

Size data and condition indices were collected for each group of animals during the setup phase of the project. Since setup, up to 11 sampling sessions have been performed at each of the five Puget Sound sites. Monitoring of the test sites began before the anticipated onset of the summer mortality events in late June and continued during each low tide cycle, approximately every 7 to 14 days. Moribund oysters and outright mortalities within each of the test plots were recorded, flagged, and documented photographically during each survey. Mortalities were counted when there was obvious shell gape, and the animals lacked any closure response. Oysters considered moribund were gaping but were able to produce a closure response.

Moribund animals were removed and preserved for histological inspection. Live specimens were collected routinely for use in the histopathology and stress protein portions of this study. The original densities of the test plots were maintained by replacing animals removed from the plots with others stockpiled in nearby plots, planted at similar densities.

Environmental Monitoring

Intertidal water and air temperatures were recorded continuously, a reading was taken every 24 to 30 min at each site beginning in mid-May 1998. Continuous DO data (readings every 15 to 30 min) were also obtained at the Mud Bay site in Eld Inlet, beginning July 11, 1998. YSI© oxygen–temperature–salinity and pH meters were used to measure these water quality parameters during selected flood and ebb tides. A water sampler bottle was

used to collect water samples at depth. Meteorological data were collected from available weather instrumentation. Tide data were computed with tide prediction software.

Phytoplankton samples were collected by fine-mesh (10-μm mesh), 0.25-m diameter ring nets at the Mud Bay and Allyn sites during each sample period. Living or formalin-fixed net samples were examined using light microscopy to generate a species list and determine species relative abundance (dominant, many, few).

An oyster condition index (CI) was derived from 11 to 31 randomly selected market-size oysters in the May to June 1998 period at the Sequim, Allyn, and Mud Bay sites. A second series of samples were taken in October to November 1998 at the above sites and in Totten Inlet. The CI was used to determine the quality or "fatness" of oysters. All CI calculations employed the gravimetric method discussed in Schumacker et al. (1998).

RESULTS

Shellfish Mortalities

Substantial mortalities of Pacific oysters occurred at the Totten Inlet, Mud Bay, Allyn Bay, and Tomalas Bay sites during the middle to late summer. Mortalities at the Sequim Bay and Eld Inlet sites were negligible.

Cumulative and average daily mortalities at four of the Puget Sound sites are shown in Table 2 for market-ready diploid and triploid oysters. Mortalities for the reported time periods ranged from 40 to 56% for triploid oysters in Totten Inlet, Mud Bay, and Allyn Bay; and 31 to 45% for diploid oysters in Totten Inlet and Mud Bay. Considerable variation was seen in seed oysters, with Totten Inlet experiencing higher die-offs than either Allyn or Sequim bay. We have limited sampling information from the Tasmanian stocks at the Eld Inlet site. Mortalities at this site over a 83-day period were 3.6% for Tasmanian Pacific oysters, 9.6% for a control stock of native Pacific oysters, and 1.4% for Pacific oysters transplanted from Mud Bay. The trend that Kittel (1998) reported seemed to be continuing.

The progression of Pacific oyster mortalities throughout the summer was followed by means of weekly to monthly field observations. Mortalities were calculated as a percentage average daily mortality between each sample period for Sequim Bay, Totten Inlet, Mud Bay, and Allyn datasets (Fig. 2). Sequim Bay did not exceed 0.04% per day at any time, and 7 out of 10 samples were less than or equal to 0.01%. The other sites experienced elevated mortalities beginning in early July in Totten Inlet and early August in Mud Bay and Allyn Bay. Totten Inlet was unusual—very high mortality occurred within the first 4 weeks of onset, mortality declined to a low level after that. Allyn and Mud bays had increasing but variable mortalities beginning later in the summer, mortalities continued at moderate levels through the end of September.

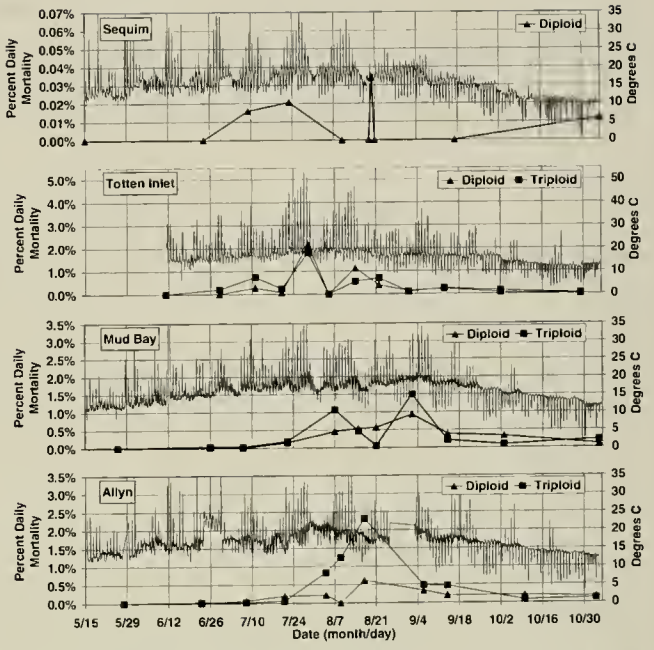

Figure. 2. Percentage daily mortality (left-hand axis) and ambient water and air temperatures (right-hand column) at Puget Sound summer mortality study sites, summer–fall 1998.

Triploid mortalities began earlier in the summer and spiked more rapidly and at a higher rate than the diploid treatments. Daily diploid mortalities did not exceed 0.6%; whereas, the average rate of triploid treatments approached 2.5%. Triploid mortalities in Mud Bay and Allyn Bay were also high; however, they tended to track the diploid die-off closely. Triploid mortalities were most pronounced in Allyn (Fig. 2). The high and sudden mortality in Allyn was unfortunate, because the commercial beds were largely planted as triploids. All triploid oysters in those commercial beds exhibited a rate and timing of mortality onset that was similar to the treatment plots.

Environmental Monitoring

All sites for the summer mortality sampling are in protected estuarine embayments. The Sequim Bay, Allyn Bay, and Mud Bay sites are in shallow heads of inlets strongly influenced by tidal exchange and freshwater input from the surrounding uplands. Water column data for Sequim Bay, Totten Inlet, Mud Bay, and Allyn Bay for the summer to fall 1998 period are shown in Table 3. High pH levels were recorded during the early summer, probably reflecting high primary productivity during this period. Salinities were depressed only for brief periods after heavy rainfall events in Mud Bay and Allyn Bay.

Temperature

Ambient temperatures were monitored at all survey sites during the 1998 sampling period (Fig. 2). Tidal period played a large role in the duration and range of recorded temperatures. Low daytime tides coupled with intense insolation resulted in high ambient air temperatures and elevated water temperatures on the incoming tide. Peak temperatures neared 53 °C (or 127 °F) and frequently

TABLE 2.

Cumulative percentage mortalities of Pacific oysters for single seed, diploid and triploid, and low- and high-density treatment groups at oyster summer mortality study sites, summer–fall 1998.

	Sequim	Totten	Mud Bay	Allyn
Days observed	197	138	184	155
Single seed	13%	65%	——	16%
Diploid low density	3.5%	45%	31%	12%
Diploid high density	2.2%	42%	42%	28%
Triploid low density	——	——	56%	40%
Triploid high density	——	50%	52%	55%

TABLE 3.

Water column conditions for all samples taken at oyster mortality study sites, summer–fall 1998.

	Temp (C)	D.O. % sat.	D.O. (mg/L)	pH	Sal. (ppt)	Secchi Depth (m)
Sequim—Surface (*n* = 3)						
Average	9.6	53.0	5.3	7.7	25.7	
Maximum	15.3	66.0	6.6	7.9	28.3	
Minimum	6.0	35.0	2.9	7.5	21.3	
Totten—Surface (*n* = 17)						
Average	18.6	98.1	7.7	8.0	28.1	
Maximum	26.5	141.4	10.6	8.5	29.5	
Minimum	12.1	67.2	5.4	7.5	26.0	
Mud Bay—Surface (*n* = 18)						
Average	19.2	71.2	5.8	7.7	24.9	
Maximum	27.4	106.0	8.6	8.6	29.1	
Minimum	10.5	39.4	3.3	7.2	19.9	
Mud Bay—Bottom to −3.75 m (*n* = 15)						
Average	17.5	74.7	6.0	7.7	28.0	1.2
Maximum	25.0	100.9	8.4	8.2	29.6	2.2
Minimum	10.7	39.4	3.2	7.3	25.9	0.6
Allyn—Surface (*n* = 13)						
Average	17.9	85.8	7.1	8.0	26.5	18.0
Maximum	22.8	115.1	9.2	9.3	29.0	27.2
Minimum	9.3	56.5	4.5	7.4	15.9	6.6
Allyn—Bottom to −3.5 m (*n* = 13)						
Average	16.8	79.4	6.4	7.9	27.5	20.0
Maximum	20.3	119.8	9.3	8.3	29.1	27.2
Minimum	10.2	59.5	4.8	7.5	23.1	15.3

exceeded 40 °C during low-tide exposure in Totten Inlet (Fig. 2). Air temperatures exceeded 25 °C during low tide at all stations between late June and early September.

Dissolved Oxygen

Dissolved oxygen (DO) concentrations recorded above the sediment surface in Mud Bay fluctuated from less than 1 mg/L (ppm) to nearly 12 mg/L. Summertime DO was closely linked with the tidal cycles. A long period of neap tides with low and slack water during the evening resulted in a daily and successive reduction in DO. For example, from September 20 through October 2, the DO was between 0.5 and 2 mg/L for about 9 days (Fig. 3). Similar, but shorter duration, declines in DO were also recorded in middle and latter parts of August. A closer look at the late September extreme low DO event revealed the following: (1) the DO was lowest at slack water; (2) if the meter was exposed (tide below about 0.3 m MLLW) the DO spiked, indicating a measurement in air; and (3) water temperature was very constant and declined only slightly in the early morning. From the second week of October onward, records from Mud Bay showed a gradual increase in DO, with numerous strong peaks (DO above 8 mg/L) corresponding to low-tide exposure to the air and circulation during strong spring tides.

Phytoplankton

Phytoplankton taxa observed in samples taken from Puget Sound summer mortality sites are listed in Table 4. A total of 32 samples were screened from June 23 to December 2, 1998. Samples taken through mid-July were composed primarily of centric and pennate diatoms and *Ceratium* spp. Beginning in mid-July and continuing through early October, *Gymnodinium splendens* and *Ceratium* spp. were typically dominant taxa, followed in relative abundance by *Chaetocreros* spp., *Coscinodiscus* spp., and *Psuedo-nitzschia* spp. Dinoflagellates declined after mid-October, and *Ditylum brightwellii*, *Coscinodiscus* spp., and *Psuedo-nitzschia* spp. were the dominant taxa.

Condition Index

Average condition indices of market-size oysters from summer mortality study sites ranged from 8.78 to 11.08 in spring 1998 (Table 5). The same size group of animals sampled in fall 1998 had

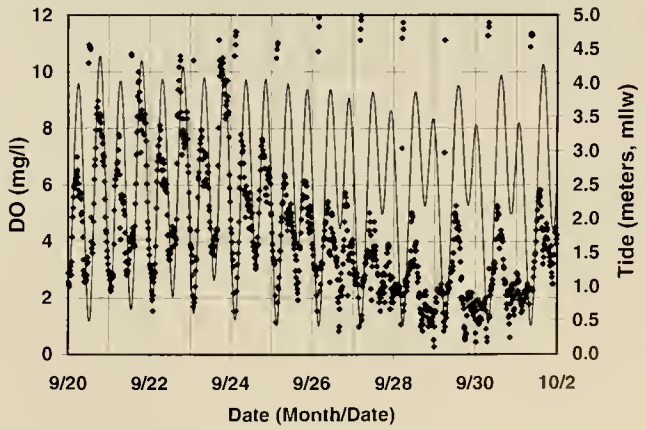

Figure. 3. Tide levels (lines) and dissolved oxygen (dots) 6 cm above the sediment surface at the Mud Bay, Puget Sound, summer mortality study site, September 20 through October 2, 1998.

TABLE 4.

Dominant phytoplankton taxa from samples taken at oyster mortality study sites, summer–fall 1998.

Diatoms
Centric
Chaetocreros spp.
Coscinodiscus spp.
Ditylum brightwellii
Eucampia zodiacus
Guinardia spp.
Rhizosolenia setigera
Skeletonema costatum
Thallassiosira spp.
Pennate
Pseudo-nitzschia spp.
Dinoflagellates
Procentrum spp.
Gymnodinium sanguineum
Noctiluca scintillans
Ceratium spp.
Alexandrium spp.
Protoperidinium spp.

average indices of 4.78 to 8.78. Triploid and diploid oysters had similar indices in the fall sampling. Allyn had the lowest average index of three sites in the spring, and over-all lowest indices in fall sampling. Many of the Allyn oysters were visibly thin and transparent.

DISCUSSION

The published literature and anecdotal reports suggest many of the mortalities occurring in Pacific oysters are the result of multiple factors or stressors, including pathogens, elevated temperatures, low DO, xenobiotic stress, and the physiological stress associated with reproduction. It is likely that multiple chronic stressors may combine to bring about mortalities and that an oyster's ability to deal with a particular seasonal stress, such as temperature, may be the deciding factor as to whether that organism will survive. Our evaluation of the first year's data from the summer mortality project supports observations reported by Glude (1975) on the rate and timing of mortality events. In addition, there seemed to be significant differences in the mortality rates among the varieties of oysters tested. Triploid oysters at the Mud Bay, Allyn Bay, and Totten Inlet test sites experienced a cumulative mortality rate 8 to 28 percentage points higher than the mortality rates of diploid oysters planted in comparable plots (Table 2). Mortality events trended toward higher rates at or immediately after neap tides when DO was lowest. Extreme air temperature spikes and increasing ambient water temperatures were also occurring at the same time. However, high temperature alone may not be lethal. Many oysters at the Totten Inlet site survived repeated exposure to temperatures over 40 °C (Fig. 2). We have not yet measured the internal temperatures of Pacific oysters in parallel with our temperature readings recorded by our data loggers. Larger oysters with thick shells, positioned vertically and partially embedded in sediment would probably have lower internal temperatures than the external (environmental) temperatures recorded by the data loggers.

A preliminary assessment of phytoplankton occurring in the study sites suggests a possible link between the densities of *Gymnodinium sanguineum*, *Ceratium* spp., *Pseudo-nitzschia* spp., and other dominant taxa with the onset of summer mortality. *G. sanguineum* was abundant in Mud Bay and Allyn Bay, and at times the water in Mud Bay had a pronounced orange-red tint. Dissolved oxygen concentrations were low and mortalities were high during this same period. These observations parallel early accounts of the interactions between oyster mortality and phytoplankton. Nightingale (1936) reported numerous occurrences of "red-tides" associated with *G. sanguineum* (densities ranged from 37 to 15,800 cells/mL). He attributed losses of Olympia oysters *Ostrea lurida* directly or indirectly to those red-tide blooms. Nightingale (1936) also assessed the effects of *G. sanguineum* in aquaria containing Olympia oysters. He used cell densities typically seen in Puget Sound blooms and observed responses ranging from excessive mucus production to shell closure and a cessation of feeding. More recent literature has associated *G. sanguineum* with fish kills and toxicity (Steidinger and Tangen 1996), and Carolyn Friedman (Bodega Marine Laboratory, pers. comm. 1998) reported seed mortalities in Tomales Bay during *G. sanguineum* blooms.

It is likely that oysters at the Puget Sound summer mortality study sites experience varying degrees of chronic stress because of the water quality and biological changes we observed at those locations. Therefore, a critical factor for survival may be the oyster's ability to elicit a satisfactory response to a suite of summer stressors. The Pacific oyster summer mortality project will continue through 2001 to describe the primary stressors affecting oyster survival, assess the effects of specific phytoplankton taxa, evaluate the role of infectious disease, and identify practical methods and tools to increase the predictability and management of mortality events. Future project reports will discuss histopatholog-

TABLE 5.

Condition indices of Pacific oysters at summer oyster mortality study sites.

		Fall 1998			
		Diploid		Triploid	
	Spring 98 Diploid	Low	High	Low	High
Sequim Bay	11.08 ± 1.99	7.92 ± 1.70	8.94 ± 2.35		
Totten Inlet		10.78 ± 1.46	9.89 ± 2.12		
Mud Bay	11.07 ± 1.81	8.25 ± 2.15	7.88 ± 2.95	8.78 ± 3.23	8.07 ± 2.17
Allyn	8.78 ± 2.83	5.39 ± 1.25	4.45 ± 1.27	4.78 ± 0.90	5.08 ± 1.32

Figures are the sample mean index values ± one standard deviation (n = 15 to 30) for diploid oysters in the spring of 1998, and diploid and triploid oysters at low and high treatment densities sampled in the fall of 1998.

ical events leading up to and resulting in oyster mortality, and physiological and biochemical responses of the animals to elevated temperatures and other stressors.

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THE EXPERIMENTAL ANALYSIS OF TIDAL CREEKS DOMINATED BY OYSTER REEFS: THE PREMANIPULATION YEAR

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ABSTRACT We report here the experimental design and observations from the premanipulation year of an ecosystem-level study investigating the hypothesis that oyster reefs control the structure and function of intertidal creeks. A group of eight tidal creeks in North Inlet, South Carolina, USA, dominated by oysters, *Crassostrea virginica* (Gmelin), were studied using a replicated BACI (Before-After Control-Incident) design in which all creeks are sampled simultaneously. Before the start of the premanipulation year, oyster biomass in the creeks was adjusted to 8 g db/m^2. Detailed geomorphological observations were made on each creek as the study began. Nutrients and chlorophyll *a* were measured weekly in each creek and exhibited seasonal and interannual influences. Intensive planktonic-microbial loop samplings were conducted seasonally and suggested a diatom-dominated winter community controlled by nutrient availability and a microflagellate-dominated summer community controlled by grazing. Nekton biomass exceeded oyster biomass in most creeks during the summer. As expected, oyster growth decreased from summer to winter, and survival was higher in winter. In the study's second, or manipulation year, the role of oysters will be tested by removing them from four creeks.

KEY WORDS: ecosystems, estuarine, oysters, creeks, plankton, nekton, field experiment, microbial loop

INTRODUCTION

Within southeastern Atlantic Coast marsh–estuarine ecosystems, tidal creeks are channels that allow the movement of organisms and materials between the marshes and mud-flats and the deeper portions of the estuary. Intertidal oyster reefs are prominent, intensely heterotrophic components of these creeks. These reefs can make an impact on biodiversity and productivity in marsh–estuarine systems by

1. providing three-dimensional (3-D) structures that increase habitat heterogeneity and supply space to support diverse assemblages of benthic and nektonic organisms (Wells 1961, Dame 1979, Tsuchiya and Nishihira 1986, Zimmerman et al. 1989, Breitburg et al. 1995, Wenner et al. 1996, Breitburg 1999),
2. modifying tidal creek morphology and hydrodynamics by structurally altering creeks, changing tidal flow patterns, and increasing water residence times (Keck et al. 1973, Frey and Basan 1978, Bahr and Lanier 1981, Lenihan et al. 1996),
3. filtering large amounts of particulate material from the water column and releasing large quantities of inorganic and organic nutrients into creek waters (Haven and Morales-Alamo 1970, Newell 1988, Dame et al. 1989, Dame 1993, 1996).

Despite numerous speculations and scaled-up estimates of the influences of filter-feeding reefs on estuaries and creeks (Cloern 1982, Officer et al. 1982, Dame et al. 1986, Newell 1988, Alpine and Cloern 1992, Ulanowicz and Tuttle 1992), no comprehensive *in situ* ecosystem-scale experiments have been performed to observe these impacts directly. We report here the experimental design and premanipulation year observations of a field study that tests the hypothesis that oyster reefs control the structure and function of tidal creeks in which they are a dominant feature. When complete, this study (known as CREEK) will have compared ecosystem-scale differences among eight tidal creeks before and after the removal of oysters from four of the creeks.

MATERIALS AND METHODS

Environmental Setting

The observations used in this investigation are from a group of tidal creeks located in the near pristine North Inlet marsh–estuarine ecosystem. North Inlet (33° 20′ N, 79° 10′ W) is located near the city of Georgetown on the northeastern coast of South Carolina, USA. The approximately 3,400 ha system is composed of salt marshes dominated by *Spartina alterniflora* (2,500 ha) and tidal creeks with intertidal oyster reefs (850 ha). A coastal maritime

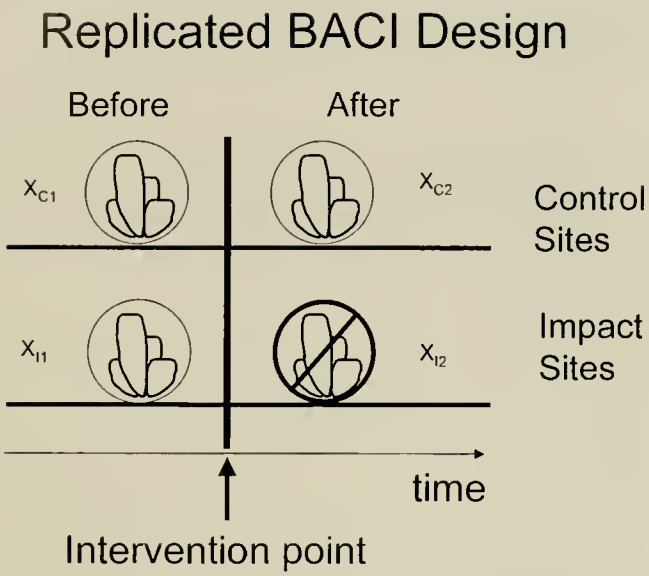

Figure 1. A graphical representation of the Before–After Control–Incident (BACI) statistical design.

forest borders the estuary and generates intermittent freshwater streamflow from approximately 1,000 ha. The climate of the area is subtropical, with average water temperatures ranging from 8 °C in January to 30 °C in July and August. The system is subject to semidiurnal tides, with an average range of 1.5 m. North Inlet has no salinity stratification and usually has very little freshwater input. Owing to shallow water depths and vigorous tidal exchange, creeks in North Inlet are well mixed and nearly always vertically homogeneous with respect to dissolved substances.

Statistical Considerations

We use a replicated BACI (Before-After Control-Impact) design (Fig. 1), with eight similar tidal creeks as replicates. Creeks were assigned to one of four blocks based on their physical locations within the estuary and suspected or known spatial differences at this scale. Blocking was deemed important because Clambank Creek creeks drain an upland area; whereas, Town Creek creeks do not border any uplands, and because there is a salinity gradient from north to south with those creeks further south more likely to experience low salinity spillover from Winyah Bay during freshets. The Before manipulation year began in March 1997 and ended in February 1998. The After manipulation year began in March 1998 following the removal of oysters from four randomly selected creeks, two each in Clambank Creek and Town Creek. Thus, the CREEK study satisfies a number of concerns raised by Hurlbert (1984): (1) there are control creeks; (2) the creeks are replicated; and (3) the creeks are sampled repeatedly, both before and after the intervention. In addition, the design heeds the recommendation of Stewart-Oaten et al. (1986) by sampling all creeks simultaneously. The statistical analysis after the intervention year is an adaptation of Stewart-Oaten et al.'s (1986) proposed analysis. This paper describes only the before or premanipulation year to highlight system variability and to identify potential sources of that variability.

Creek Geomorphology

The eight tidal creeks used in this study are located on two larger order creeks, Town Creek and Clambank Creek, and are within 1 km of each other (Fig. 2). The observed creeks are typically ephemeral (i.e., dry at low tide). A detailed topographic–bathymetric survey of each creek and its basin was conducted

Figure 2. An areal depiction of the study area.

TABLE 1.

Structural dimensions of the eight experimental tidal creeks.

Dimension	Creek							
	1	2	3	4	5	6	7	8
Length (m)	177	101	164	229	138	232	174	423
Length w/tributaries (m)	359	169	284	254	153	306	309	517
Width at mouth (m)	7.6	5.8	6.7	4.6	4.9	3.1	6.7	9.5
Cross-sectional area at mouth (m^2)	5.4	3.2	4.7	2.5	2.5	2.6	4.8	5.7
Water volume (m^3)	667	321	527	520	446	391	623	1,423

utilizing a Topcon total station. All elevations were referenced to a common datum that, in turn, was referenced to eight U.S. Geological Survey permanent benchmarks. The data were used to estimate creek length, width, cross-sectional area at mouth, surface area, and water volume.

Physical and Chemical Variables

Beginning in early March 1997 and continuing until late February 1998, water samples were taken once a week from each study creek for chemical analysis. The samples were taken approximately midway between the daytime high and low tide stages. Water samples were taken from the center of each creek mouth at a depth of 1 m below the surface but not closer than about 0.3 m from the bottom. Triplicate samples were collected from each creek, and all creeks were sampled within 45 min. The samples were immediately placed in ice and rushed to the laboratory for analysis. Temperature was measured at each site as samples were collected. Salinity and concentrations of ammonium, nitrate + nitrite, orthophosphate, and chlorophyll *a* were determined using standard techniques. All water chemistry parameters were logarithmically transformed to ensure equal variability, but temperature and salinity observations were not transformed. The Grubbs test (Grubbs and Beck 1972) was used to determine outliers; only two observations were deleted.

Plankton and Microbial Loop Observations

The planktonic food web in the experimental system was examined using a series of bioassay experiments. These studies were conducted at a morning mid-ebb tide on five dates (March 20, June 13, July 27, September 13, and December 9) in 1997. Replicate samples were collected at each of the eight experimental creeks and dispensed into 1-L acid-cleaned polycarbonate bottles. Samples were incubated under various treatments designed to examine the effect of substrate enrichment or reduced grazing pressure on phytoplankton community biomass (chlorophyll *a*). The treatments included 4 μm NH_4 addition, 20 μm glycine addition, and a 20:1 dilution treatment used to reduce grazing pressure on phytoplankton by decreasing encounter rates between microzooplankton and phytoplankton prey (Landry and Hassett 1982). Lewitus et al. (1998) have found, from experiments involving serial

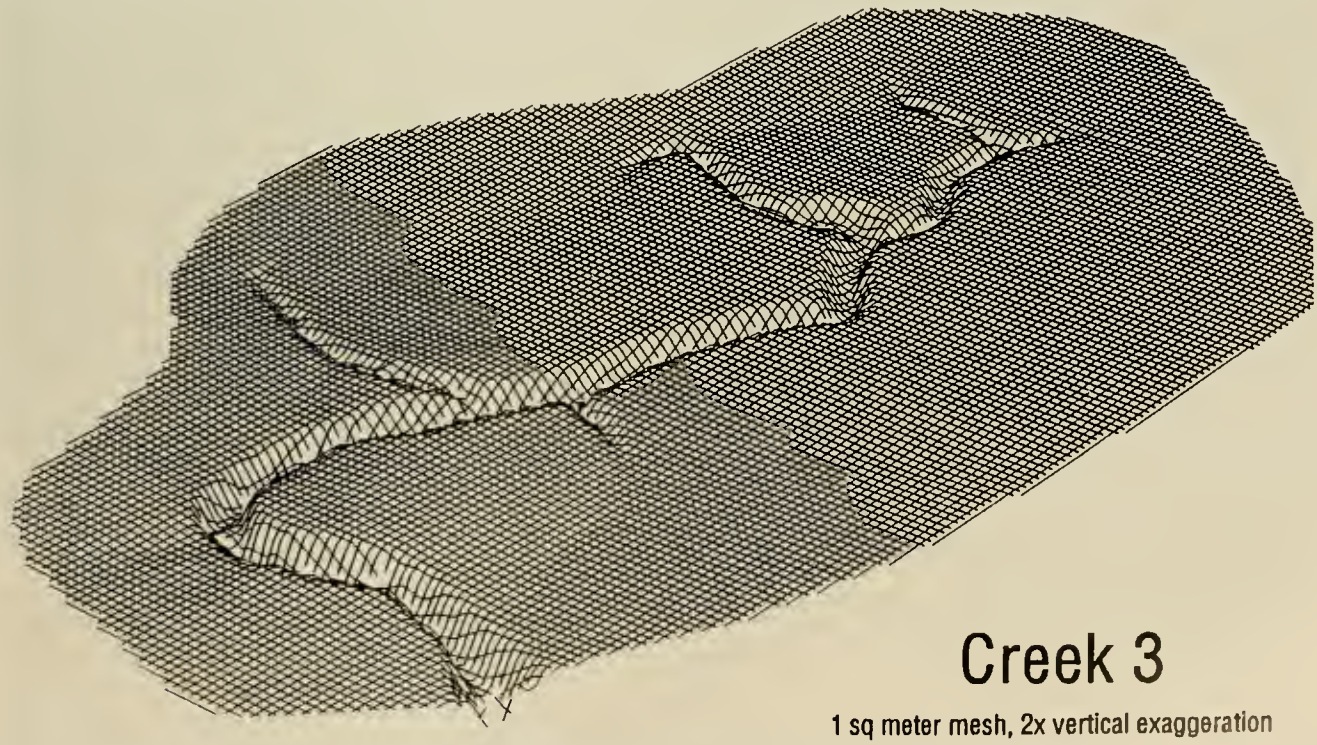

Figure 3. An example of a 3-D surface plot for Creek 3 showing geomorphological relief.

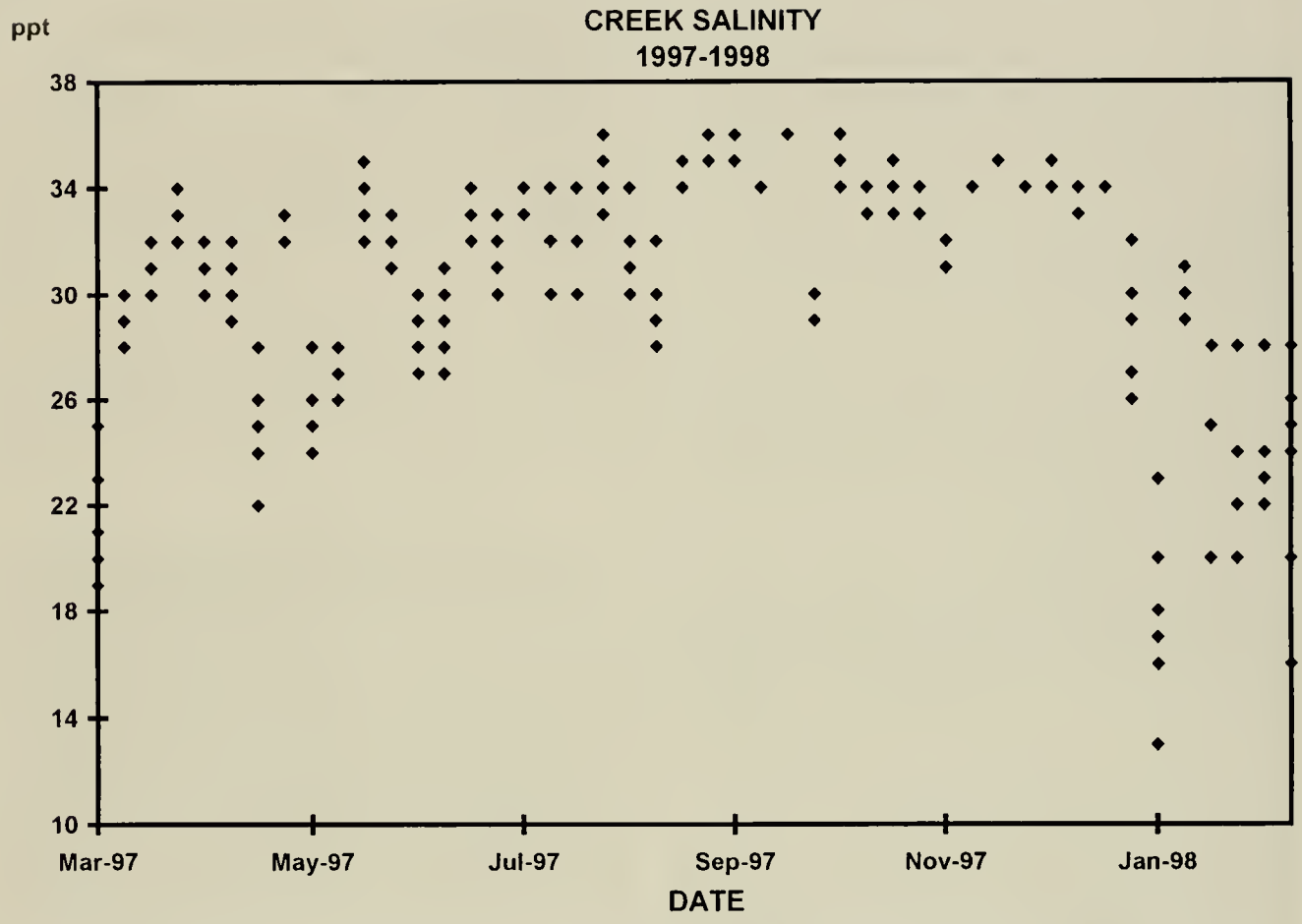

Figure 4. Time plot of salinity data (ppt) from the eight experimental tidal creeks during the premanipulation year of 1997 to 1998.

dilution of North Inlet water, that a 20:1 dilution fell within the range where grazer reduction over 72 hours was saturated. Bottles were incubated in raceways containing flowing estuarine water to simulate tidal creek temperatures. Overhead fluorescent cool white bulbs provided uniform irradiance adjusted to a light–dark cycle simulating natural conditions. Water samples were mechanically stirred (gently) at uniform rates between bottles. Chlorophyll *a* was measured daily at midday over the 72-hour time course.

Nekton Abundance and Biomass

Nekton abundance and biomass were determined for each creek in March, June, August, and November of 1997. Simultaneous collections of nekton were made with block nets set at early morning slack high tide at all eight creek mouths. Catches were removed from the block nets, and pools within each creek bed were seined at low tide to provide a complete assessment of fish and motile macroinvertebrate use of the creeks. All samples were frozen and subsequently sorted to the species level. Total abundance and biomass were determined for each species. Up to 100 individuals of each species were measured to the nearest 2.0 mm. Total wet weight biomass per cubic meter of water volume was the primary unit used to compare catches from creeks with different volumes. A factor of 0.25 was used to convert wet weights to dry weights (Caspers 1957).

Oyster Biomass, Growth, and Survival

Before the premanipulation year observations began, oyster biomass in each creek was estimated from 10 quadrats (0.25 m^2) distributed at different elevations along the length of the creek. Oyster biomass in each creek was adjusted to an average of 8 g dry body weight/m^3 of water. The grams dry body per m^3 relationship was used, because it more realistically describes the benthic–pelagic coupling of the oysters to the water column (Dame 1993). During the premanipulation year, oyster growth and survivorship were observed by placing plastic-mesh bags containing 25 marked and measured oysters in each of the eight experimental tidal creeks. Because the creeks are ephemeral, and tidal exposure is a critical factor in bivalve physiology, bags were placed at four locations approximately equidistant along the mainstem of each creek at approximately the same measured elevation. Summer observations were made between July and October and fall—winter observations were from October to February. Growth was measured as change in length, measured to the nearest 0.1 mm. Biomass to length relationships determined by Dame (1972) were used to calculated dry body biomass.

RESULTS

Creek Geomorphology

The detailed topographic–bathymetric survey of each of the experimental creeks was used to generate longitudinal profiles, hypsometric curves, storage curves, and 3-D surface maps. The results of these surveys are summarized in Table 1, and an example of a 3-D surface map is given in Fig. 3. The creeks range in length, including branches, from 153 to 517 m, and bankfull water volume

ranges from 231 to 1,423 m^3 (Table 1). The surface-t- volume ratio by creek ranged from 1.3 to 2.6. There was less than an order of magnitude difference for any measure between creeks.

Physical Environment

Temperature ranged from 8 to 35 °C and displayed a well-defined seasonal trend. Analysis of variance (ANOVA) of all temperature observations shows that date (time) explains 99.2% of the variation in these data ($P < 0.01$). Salinity data ranged from 12 to 36 ppt (Fig. 4). There was a distinct period of lower and more variable values in all creeks during the December 1997 to March 1998 period. While 91.1% of the variability in the salinity observations were attributed to date, a significant ($P > 0.01$) amount of variability (1.1%) was attributable to creeks.

Water Chemistry

Chlorophyll *a* concentrations range from near zero in the winter to about 42 μg/L in the summer, and depict an annual curve when plotted (Fig. 5). Results of the ANOVA of the premanipulation year data show that 91.6% of the total variability was attributable to time of year and 3.8% was related to creek differences. There were significant differences among dates and blocks of creeks, but not among creeks within blocks. Ammonium concentrations are shown in Figure 6. Values are near zero in autumn and winter and are near 7 μm/L in spring and summer. ANOVA revealed that about 84% of the variability was attributable to date and 4.4% was attributable to creek. Blocks of creeks were not significantly different; however, there were cases where creeks within blocks were different. Phosphorus concentrations were low (near zero to about 1.5 μm/L), with maximum values in summer and minimum in winter. Concentrations of nitrate + nitrite were also low (near zero to 2.7 μm/L), with maximum values and variability in winter and spring. ANOVA showed that variability of nitrate+nitrite and phosphate was similar, although less of the variability in nitrate+nitrite was explained.

Plankton

The seasonal patterns exhibited by chlorophyll *a* in response to NH^4 addition, glycine addition, or dilution were generally similar among creeks (Figs. 5 and 6). For water collected from March to July, the addition of NH_4 occasionally stimulated chlorophyll *a* concentrations in the water from Clambank tributaries (creeks 1–4) but not Town Creek tributaries (creeks 5–8), which is consistent with the lower ambient NH_4 concentrations in the former group (Fig. 7). During the summer months, the Town Creek tributaries averaged higher concentrations of NH_4 than those of the Clambank

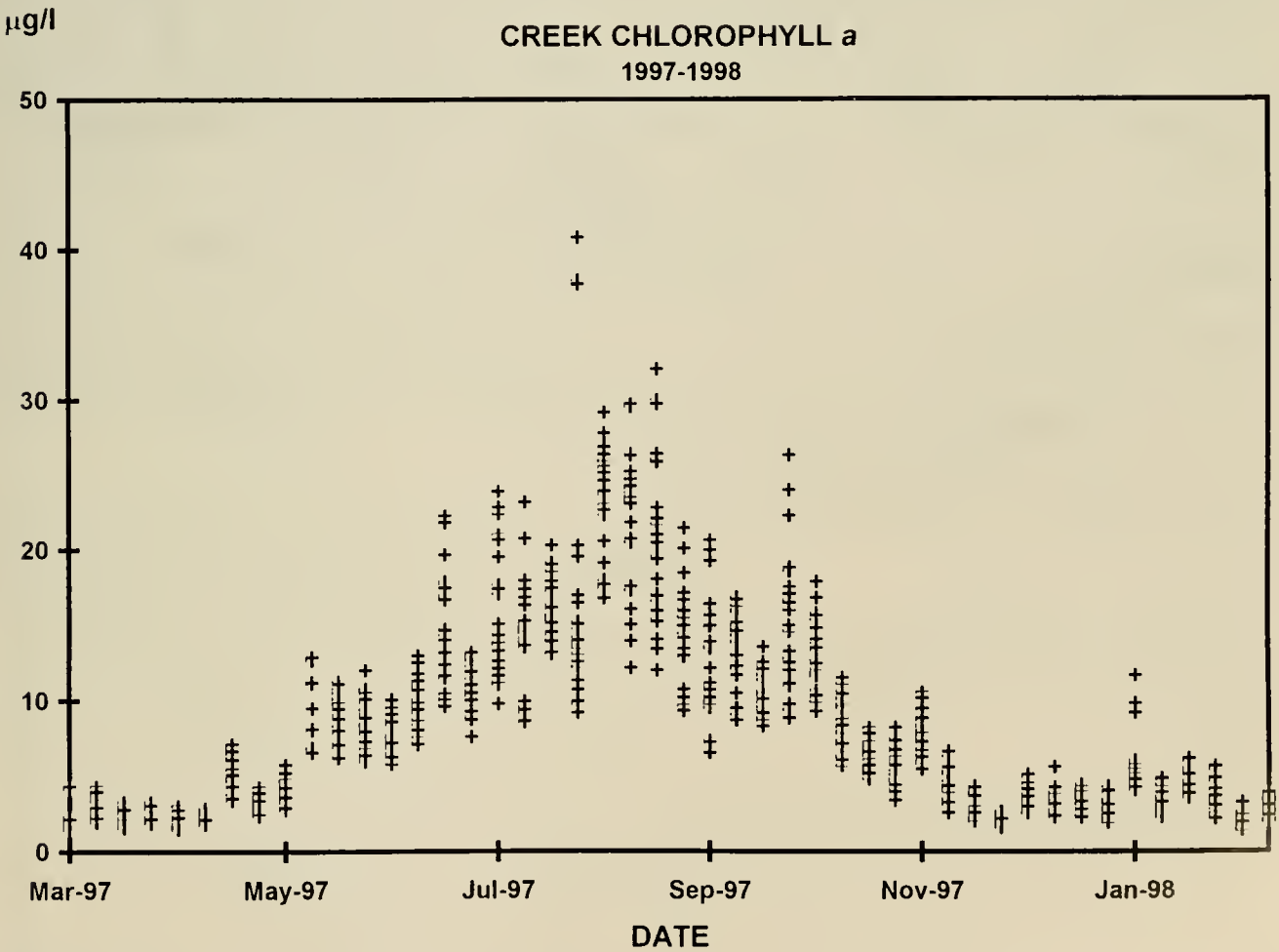

Figure 5. Time plot of chlorophyll *a* concentrations (μg/L) from the eight experimental tidal creeks for the premanipulation year of 1997 to 1998. (triplicates shown).

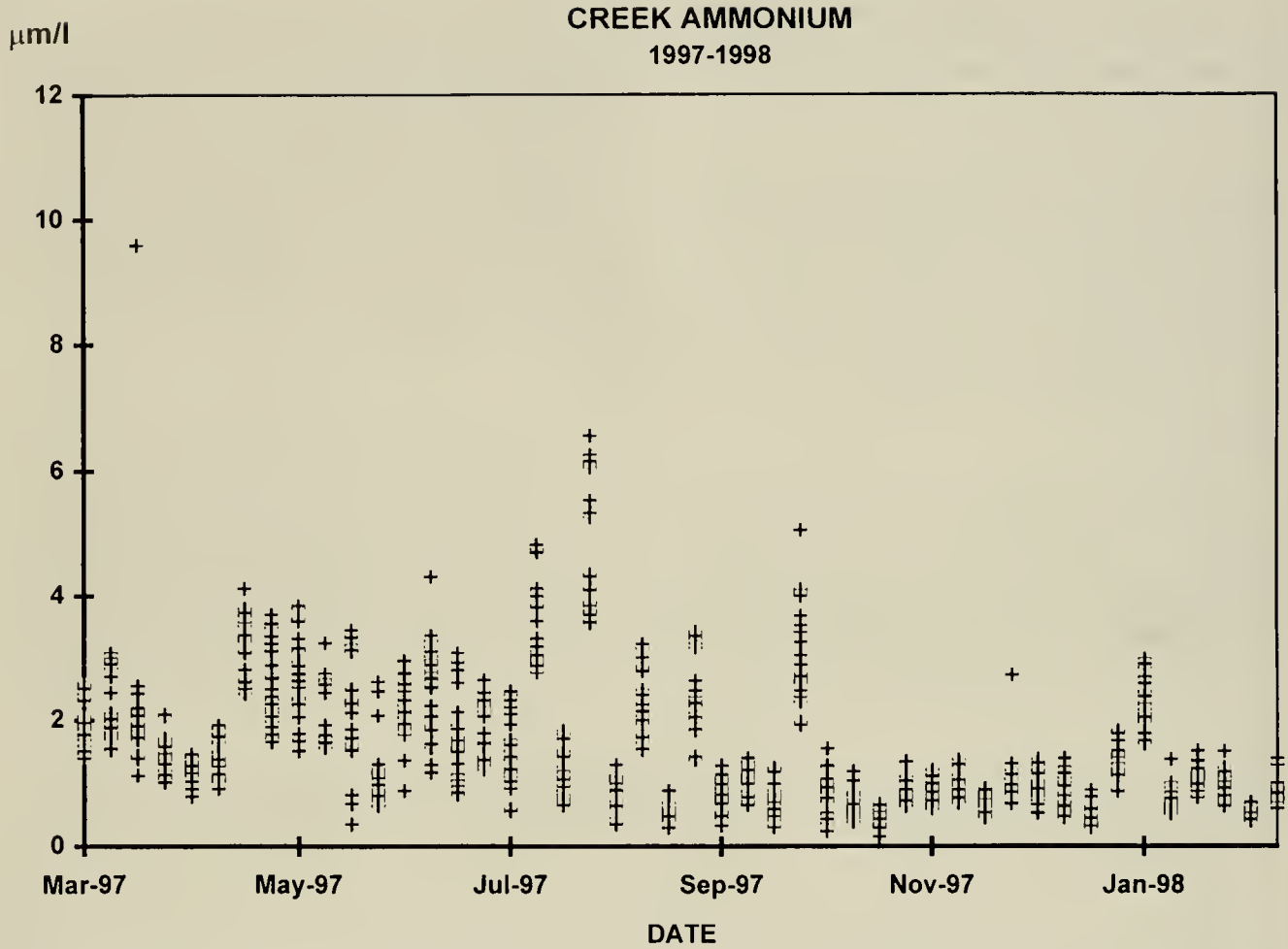

Figure 6. Time plot of ammonium concentrations (µm/L) from the eight experimental tidal creeks for the premanipulation year of 1997 to 1998. (triplicates shown).

tributaries. Dissolved organic carbon (DOC) concentrations were lower in the Town Creek group during this same period.

In almost all cases, the dilution treatment led to greater stimulation of chlorophyll production than either of the substrate additions. The dilution effect was greatest in the summer when nanophytoplankton dominated the community and minimal during the winter and early spring when a microplanktonic diatom-dominated community regulated by nutrient availability was prevalent (Fig. 8).

Oysters

Oyster growth was higher in summer than in winter and was not significantly different between creeks (Fig. 9). Oyster survivorship was higher in winter than summer, and no significant differences between creeks were evident (Fig. 10).

Nekton

Seasonal variations in total nekton biomass were consistent among creeks. Biomass was highest in all creeks during the summer (Fig. 11). Lowest biomasses were observed in March, when water temperatures and salinities were lowest. During the summer months, nekton biomass is generally equal to or as much as five times greater than oyster biomass; whereas, the reverse is true in winter. More than 60 species of fishes, shrimps, and crabs (mostly young of the year) were identified. Seasonal shifts in species dominance were strong and consistent among creeks. Each season, the same creeks supported the highest or lowest nekton densities. These differences seem to be related to geomorphological differences among the creeks.

DISCUSSION

The choice of the appropriate statistical methods for detecting trends and perturbations in ecosystems has been a lively area of debate in the ecological literature (Hirsch et al. 1991). Green (1979) proposed a multifactor ANOVA approach for detecting changes in a single stream for which there are observations upstream and downstream of the perturbation. Green's design was criticized by Hurlbert (1984) for lacking spatial and temporal replication. Hurlbert introduced the term pseudoreplication to describe multiple measurements of a single study site versus what he considered true replication: measurements of multiple study sites at multiple time points. Stewart-Oaten et al. (1986) refined Green's (1979) approach by proposing that the difference between control and impact or manipulated sites measured at multiple time points before and after the manipulation was the appropriate response variable for analysis. They called this approach BACI.

Although salinity observations seem to provide evidence of the strong interannual ENSO climatic event of 1997 to 1998, the temperature data only reflect a normal seasonal pattern. ENSO events

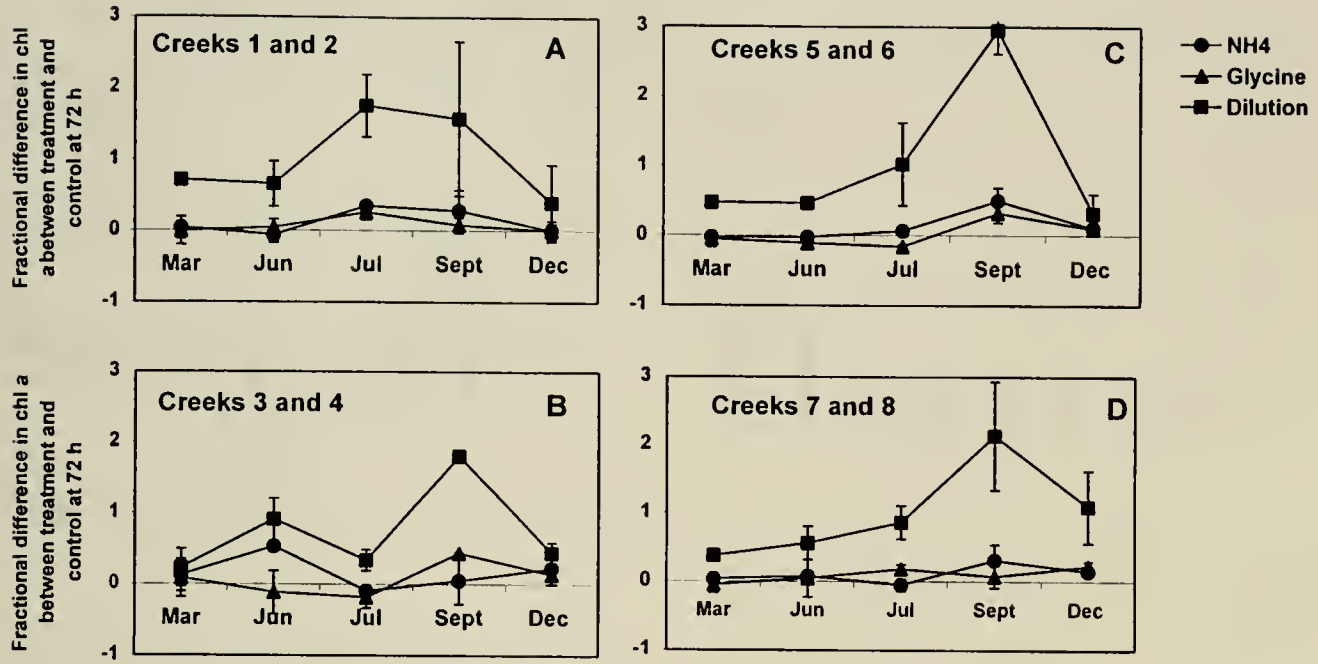

Figure 7. Results of addition and dilution incubation experiments on the planktonic community in the eight experimental creeks during the premanipulation year 1997 to 1998. A value of 0.0 indicates no difference in chlorophyll *a* between experiment and control after 72 hours of incubation, and a value of 1.0 indicates a 100% increase in chlorophyll *a* in the experiment.

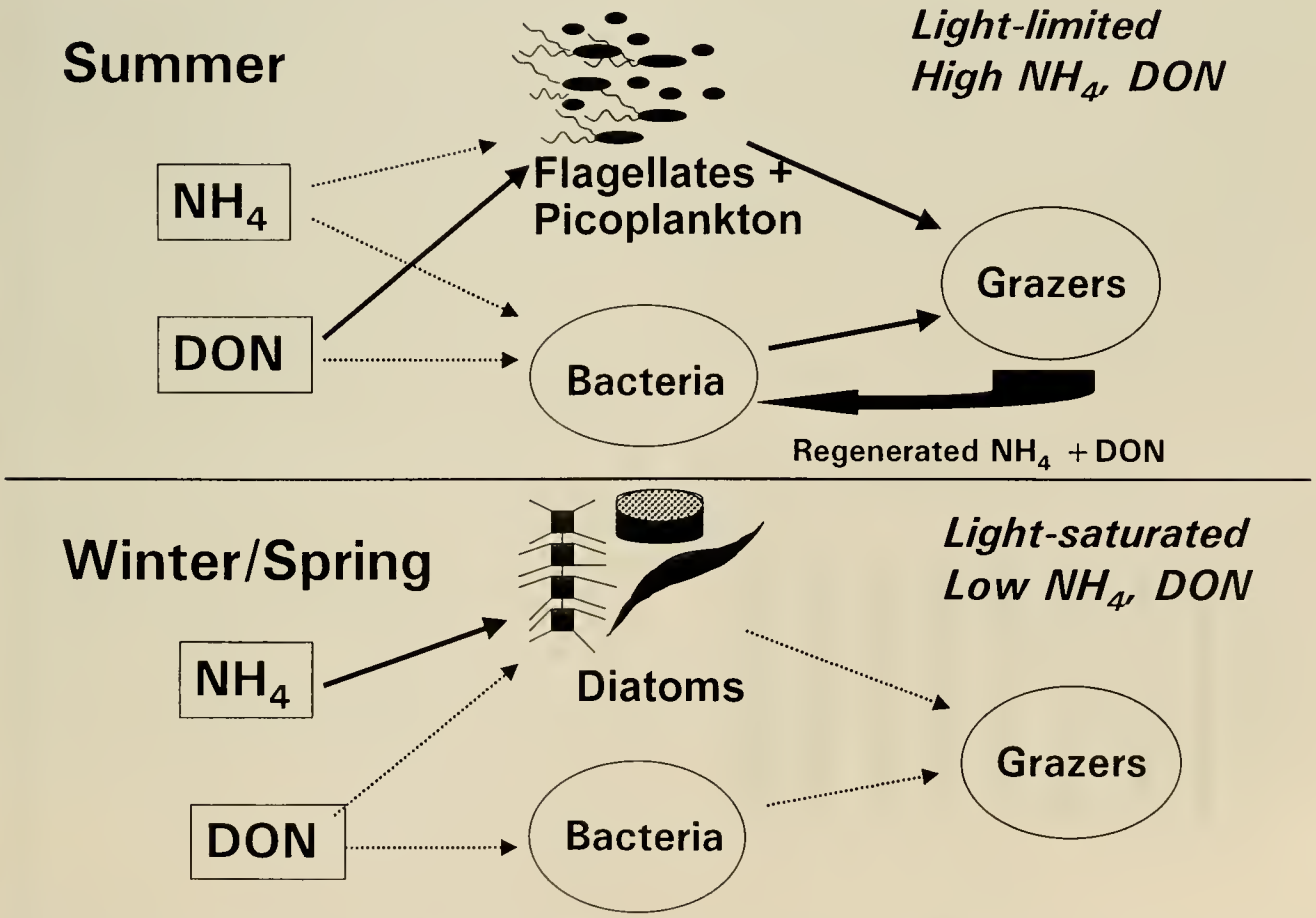

Figure 8. A graphical representation of the two different states of the planktonic web within the premanipulated creeks. Solid arrows indicate stronger relationships.

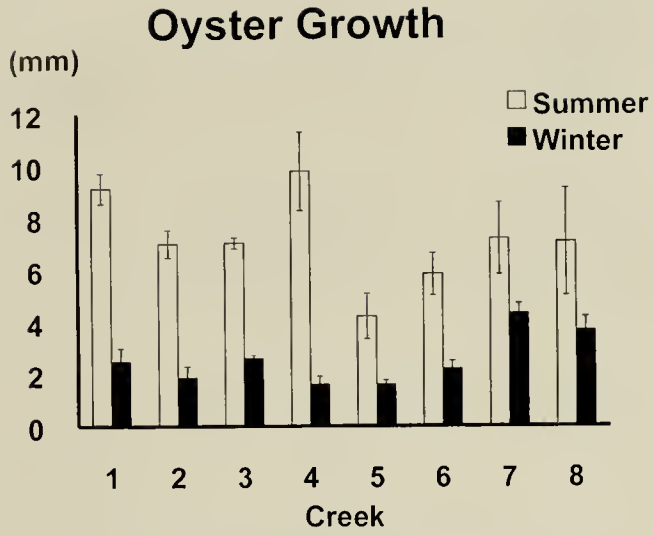

Figure 9. Oyster growth in the experimental creeks during winter and summer seasons in the premanipulation year 1997 to 1998.

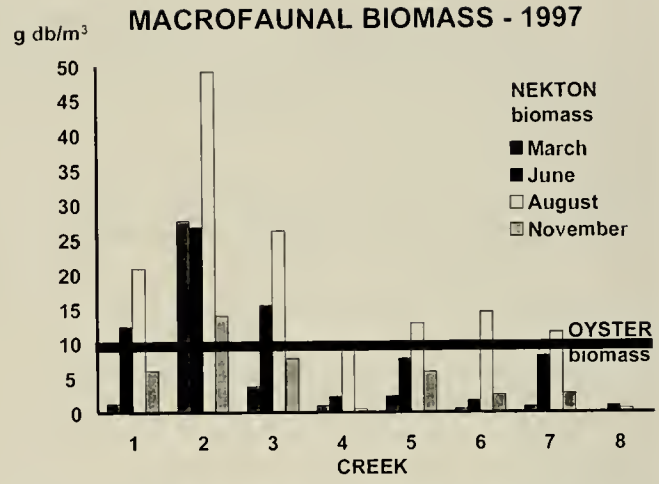

Figure 11. Seasonal variations in nekton biomass (g db/m^3) in the eight experimental creeks during the premanipulation year 1997 to 1998.

are thought to influence the southeastern Atlantic coast by increasing winter precipitation and decreasing air temperatures (Ropelewski and Halpert 1986, Philander 1990, Hanson and Maul 1991). Although higher than normal freshwater discharge to southeastern estuaries with a higher groundwater table should be expected during these events, these features were only statistically observed from the northeastern coast of South Carolina to Florida (Kuhnel et al. 1990). During the 1997 to 1998 El Niño, the coastal zone of the Carolinas received over 200% of normal precipitation for this period. In North Inlet, a normally euhaline estuary, the increased rainfall depressed salinities below 15 ppt for one 3-week period during the December 1997 to March 1998 period. It is probably this precipitation that depressed salinities and increased the variability of our salinity observations during the winter period.

Chlorophyll *a* concentrations are a measure of the major phytoplanktonic food source of the dense oyster populations within North Inlet. The data in Figure 5 are similar to those reported previously for North Inlet (see Lewitus et al. 1998). There seems to be a seasonal transition in the microbial food web structure and regulation, from a microplanktonic diatom-dominated community regulated by nutrient supply during the winter to a nanoflagellate-prevalent phytoplankton bloom regulated by microzooplankton grazing in the summer (Fig. 7). The generally consistent lack of a nitrogen response by the phytoplankton strengthens the possibility that oyster removal may lead to nitrogen limitation.

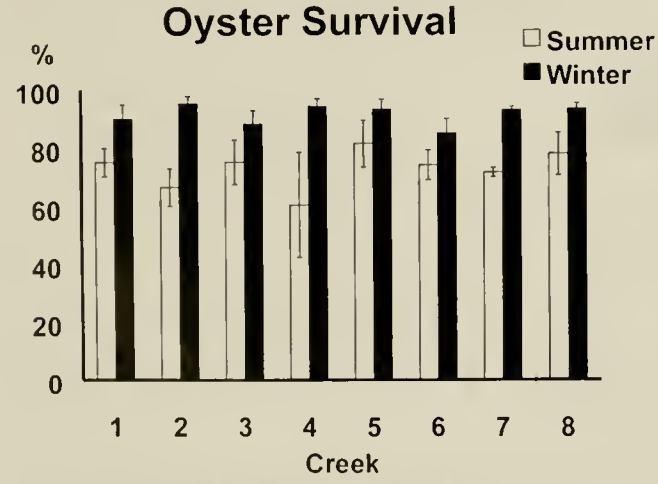

Figure 10. Summer and winter oyster survival in the experimental creeks during the premanipulation year 1997 to 1998.

Ammonium is the major inorganic nitrogen source in North Inlet, because the estuary is a bar-built, high-salinity salt marsh with little freshwater input. This form of inorganic nitrogen is also a major excretory product of oysters and, thus, a major component in the regeneration of nitrogen in this system (Dame 1993). Nitrogen is typically limiting in many coastal and estuarine systems (i.e., there is an inverse relationship between nitrogen and chlorophyll during the bloom period); however, this is not the case in North Inlet. In historical data from North Inlet (Lewitus et al. 1998), and in the data presented here, there is a positive correlation between chlorophyll *a* (Fig. 5) and ammonium (Fig. 6) concentrations. This relationship suggests that ammonium is not limiting during the summer bloom and that other factors, such as grazing, are important (Lewitus et al. 1998). Concentrations of nitrate + nitrite are elevated during the same winter period as reduced salinities, suggesting terrestrial runoff as a source and nutrient limitation as a control on the winter phytoplankton.

Although nekton use of the intertidal creeks was not uniform in space or time, the occurrence of spatially stable patterns suggests that temporally stable features of the physical habitat were important determinants of use. Geomorphological and associated hydrographical features of intertidal creeks may be key attributes of habitat quality as it relates to the nursery function of salt marsh channels. High nekton biomass may, in turn, exert significant influences on water quality criteria that affect the creek system (i.e., NH^4 concentrations). The equivalent magnitudes of nektonic and oyster biomass within these tidal creeks implies that there may be a much more complicated and hitherto unforeseen control of nutrient cycling within these systems.

The premanipulation year studies reported here show that: (1) annual and interannual environmental cycles are evident; (2) no two creeks are the same, but they are all similar in that 85% of the observed variability was explained by date; (3) the plankton community exists in two states, which are regulated differently; (4)

oyster growth and survivorship are normal; and (5) nekton are much more important than previously thought and may match oysters in their influence on tidal creek systems. These observations will certainly be elucidated by the results of the postmanipulation (oyster removal) year.

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OYSTER REEF RESTORATION: CONVERGENCE OF HARVEST AND CONSERVATION STRATEGIES

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ABSTRACT Oyster reef restoration, protection, and construction are important to meeting harvest, water quality, and fish habitat goals. However, the strategies needed to achieve harvest and conservation goals have often been considered to be at odds. We argue that these goals are, in fact, compatible and that the same strategies will promote a sustainable harvest of the resource, increased filtration of estuarine waters, and increased provision of structured habitat for finfish, crabs, and other organisms that utilize oyster reefs or receive benefit indirectly from them. Creation or designations of unharvested sites (refuge sites) are key components of these strategies. Unharvested reefs have the potential to provide vertical relief, which is typically destroyed by harvest practices, to act as a source of larvae, which potentially increases the supply of harvestable oysters, and to protect those individuals most likely to have some resistance to disease. Furthermore, proper monitoring and design of refuge and restoration efforts are critical to providing information needed to improve the success of future restoration efforts, and will simultaneously enhance the basic information needed to understand the ecology of oysters and their role in estuarine and coastal systems.

KEY WORDS: oyster reef, restoration, water quality, harvest, fish habitat, *Crassostrea virginica*, sanctuaries

INTRODUCTION

Oyster reef restoration is a recognized need by resource agencies in most states along the Atlantic and Gulf of Mexico coasts of the United States. In general, the initial impetus for these programs has been declining harvests and standing stocks of oysters that are at an all time low (MacKenzie et al. 1997a, MacKenzie et al. 1997b, Luckenbach et al. 1999, Coen and Luckenbach 2000 and references therein). Although numerous factors have been implicated in these declines, a consistent factor has been the destruction of reef habitat during the harvesting process (Hargis and Haven 1999, Lenihan and Micheli 2000). To date, most oyster restoration programs have focused on improving oyster habitat as a means of enhancing the commercial fishery (Luckenbach et al. 1999, Coen and Luckenbach 2000). Harvest of oysters involves removal of the reef substrate and, therefore, a decrease in available settlement and growth habitat for subsequent recruits to the oyster population. In addition, most harvesting practices are destructive to the reef matrix, reducing the vertical relief and damaging structural integrity in excess of that caused by removal of the individual oysters actually marketed (Hargis and Haven 1999, Lenihan and Micheli 1999). Shell repletion programs attempt to mitigate this habitat removal and destruction by adding shell as substrate for settlement of oyster larvae. A consequence of these repletion efforts has been a shift toward put-and-take fisheries (Coen and Luckenbach 2000).

Recognition of oyster reefs as valuable estuarine habitats that provide a range of ecosystem services is increasing (Coen and Luckenbach 2000, Coen et al. 1999b). The original goal of restoring and enhancing fishery stocks has been augmented, and in a few instances, superceded, by two additional goals: (1) improving water quality (by removing a portion of the phytoplankton standing stock) and (2) providing a structured habitat that may increase

secondary production, including production of finfish and decapod crustaceans, such as crabs (Fig. 1) (Wenner et al. 1996, Coen et al. 1999a, Coen 1999b). Extrapolations from laboratory filtration rates (Newell 1988, Powell et al. 1992), direct field measurements (Dame 1996 and references therein), and ecosystem-level modeling (Ulanowicz and Tuttle 1992) have clearly demonstrated that oyster reefs can have significant impacts on material processing and energy flow in estuarine systems. The recognition of the importance of oysters' ability to reduce phytoplankton biomass as a result of their filtering capabilities coincides with an increased concern over eutrophication in coastal waters. Increased anthropogenic loadings of nutrients make the ecosystem-level role of suspension feeders (such as oysters) all the more critical at the same time that overharvest and disease have reduced populations through much of their range.

Furthermore, descriptive and experimental studies have pointed to the importance of oyster reefs as habitat for commercially and ecologically important finfish and decapod crustaceans (see Wells 1961, Bahr and Lanier 1981, Stanley and Sellers 1986, Breitburg 1992, Breitburg 1999, Wenner et al. 1996, Coen et al. 1999a, Coen 1999b, Harding and Mann 1999). Although few specifics are known about the relationships among oyster reef structure, oyster population structure, and the provision of these ecosystem services, it is likely they are related to the vertical relief of reefs, the size and numbers of reefs, the overall estuarine habitat landscape, habitat health, and the population density and age structure of oyster populations. Seemingly, this sets up a conflict between the goals of fisheries exploitation and those of ecological restoration and conservation. With recent revisions to the Magnuson–Stevens Fishery Conservation and Management Act (1996) this conflict might be expected to intensify (Coen et al. 1999b).

In this paper, we address the challenge of simultaneously achieving all three goals of oyster reef restoration (fisheries, water quality, and habitat), highlight ecological processes that may make the feasibility of meeting all three goals more or less difficult, and discuss the potential benefits of melding research and restoration activities. We emphasize our belief that these goals are generally compatible and the importance of keeping all three goals in mind to achieve sound habitat and resource management and restoration. Many of the ideas in this paper stem from discussions at the special session and workshop on oyster reef restoration organized by L. Coen and M. Luckenbach at the 2nd International Conference on Shellfish Restoration held in Hilton Head, South Carolina, in November 1998. Our intent is to summarize some of the major themes and explore the constraints associated with sustaining the goals of fisheries exploitation and habitat conservation, not to provide a comprehensive review of the workshop and presentations or to address all of the issues related to oyster restoration raised therein.

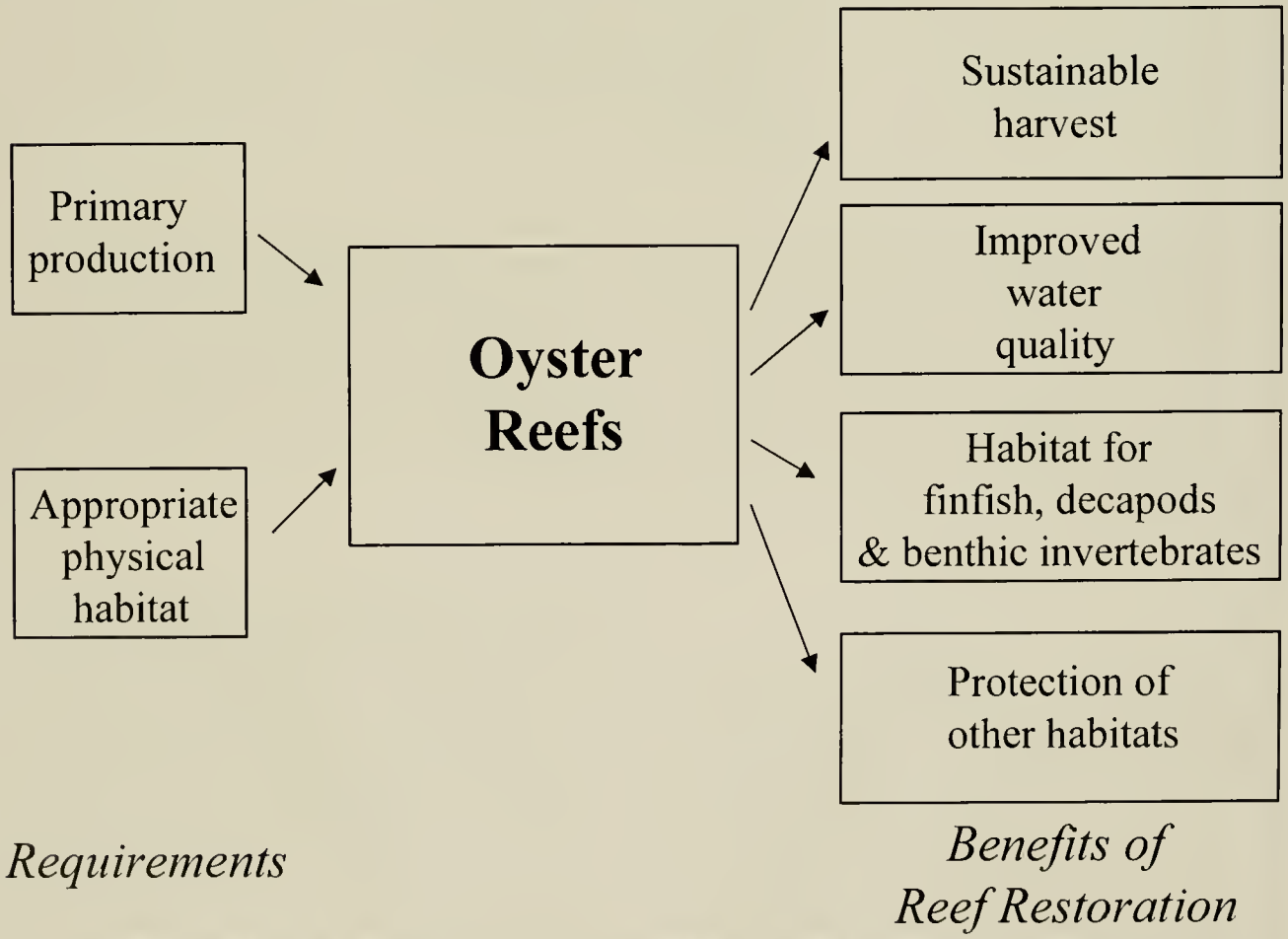

Figure 1. Restoration of oyster reefs has three primary goals: increasing sustainable harvests of oysters, improving water quality through the removal of phytoplankton biomass, and increasing structured habitat utilized by finfish, crabs, benthic invertebrates, and (especially for intertidal reefs) birds. In addition, studies by Meyer and colleagues indicate the possibility that oyster reefs can play a significant role in reducing shoreline erosion and protecting salt marsh habitat (see Meyer et al. 1996, Meyer et al. 1997).

Although many areas of uncertainty remain, we believe a pattern of convergence is emerging (see recent reviews by Lenihan and Peterson 1998, Coen et al. 1999b, Luckenbach et al. 1999, Coen and Luckenbach 2000).

COMPATIBILITY OF HARVEST AND ECOLOGICAL GOALS OF OYSTER REEF RESTORATION

Are sustainable harvest and ecological goals of oyster reef restoration compatible? The relationships between production and biomass, as well as between the fishery and ecological benefits of unharvested refuge areas, contribute to our belief that the answer is yes. Figure 2 illustrates the possible relationships between production and biomass. Maximum production of a resource is achieved at a biomass lower than the maximum potential biomass because of processes ranging from self-shading in phytoplankton, to age-dependent growth declines, to prey depletion that occurs at high population densities of consumers. In part, the degree to which harvest and ecological values of reefs coincide will depend on which of the family of curves depicted in Figure 2 best describes estuarine oyster populations. Maximum sustainable yield strategies in fisheries generally focus on keeping a population near its maximum rate of production but on the descending portion of the curve (i.e., biomass greater than that at maximum production), where overharvesting of the resource is less likely to occur than along the ascending portion of the curve (see Applegate et al.1998, Restrepo et al. 1998 for a comprehensive discussion of these curves in a fishery management context). Because maximum filtration rates and maximum production are both related positively to per capita growth rates (Powell et al. 1992, Hoffman et al. 1995), population densities producing high levels of sustainable harvests should also be those that lead to a high (possibly maximal) ecological benefit of water filtration by oysters. Finally, although less well understood, we argue that "more is better" in terms of the habitat oysters provide for fish, crabs, and other benthic organisms, but, as with the other goals, there is a decreasing benefit portion of the curve. Something short of complete coverage by oysters is needed to produce a diversity of benthic habitats that includes soft bottom, submerged aquatic vegetation, salt marsh, oyster reefs, and clam beds, where these have naturally or historically co-occurred. As important, many fish and decapods orient toward the edges of reefs and do not simply utilize the large interior areas (Powell 1994, Breitburg 1999). It is critical to keep in mind that even if the optimal biomass for harvest and ecological goals do not coincide precisely, movement toward all three goals requires increasing oyster biomass in most estuarine systems.

The more the production versus biomass curves are skewed to the right (e.g., curve C rather than curve A in Fig. 2), the higher will be the optimum oyster standing stock for a sustainable fishery and the greater will be the coincidence between biomass levels optimizing the filtration capacity of the oyster population and the provision of habitat for other biota. Several features of oyster biology, as well as ecological interactions among oysters, the

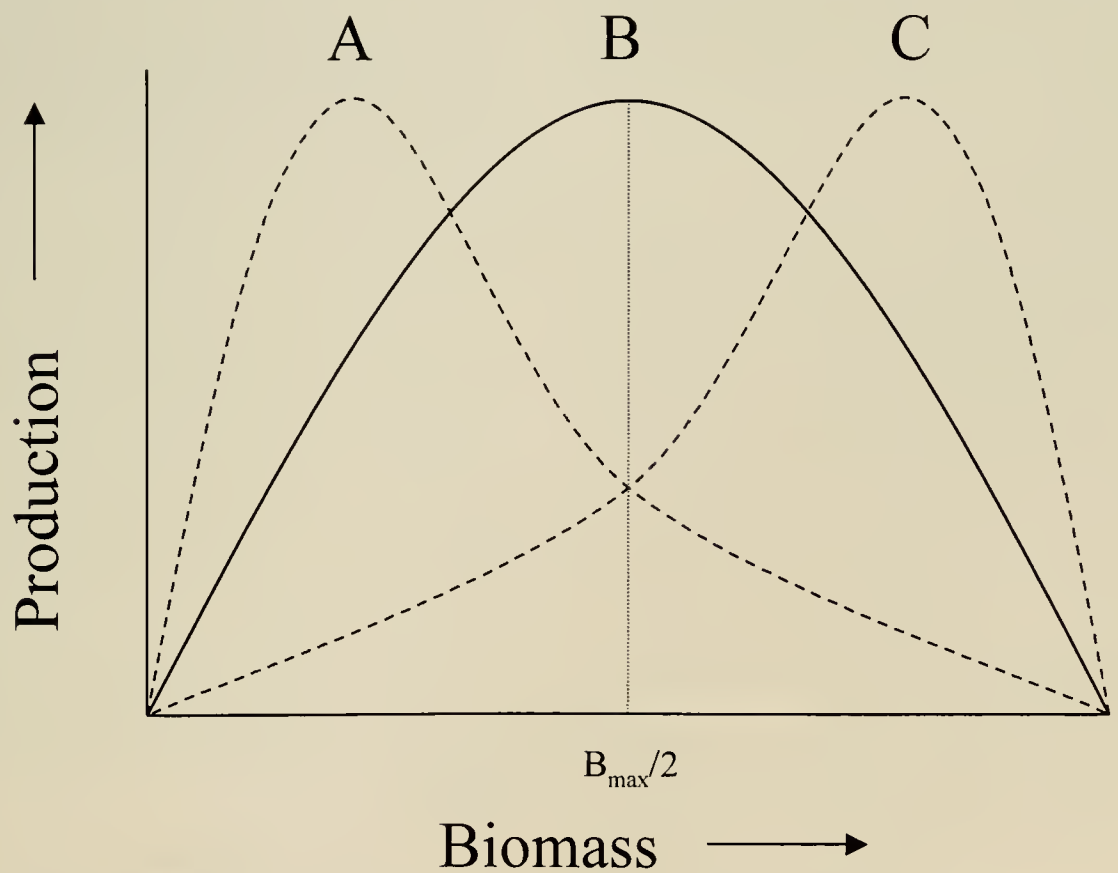

Figure 2. Relationship between production and biomass. Theoretical considerations suggest that maximum production will often occur at one half the maximum biomass (Applegate et al. 1998). However, interference competition and resource depletion can skew the curve to the left (A), and increased efficiency or reproductive success at high densities can skew the curve to the right (C). We suggest that under most conditions, oyster populations will be described by curves B or C, making harvest, water quality, and habitat restoration goals compatible.

physical environment, and other biota suggest a high-biomass–high-productivity relationship, with greatest success for all three goals occurring with well-developed or "mature" high-relief reefs. High density within oyster beds is likely the optimal condition for the oysters themselves, because the preferred settlement substrate for oyster larvae is oyster shell (e.g., Hidu 1969, Luckenbach et al. 1997, Bartol and Mann 1999), the fertilization success of sessile animals is increased at high densities (Levitan 1991, Levitan et al. 1992), and the subtidal reefs will maintain greater vertical relief, reducing sedimentation effects and enhancing local flow rates (Lenihan and Peterson 1998). High aerial coverage by oysters should provide insurance against the strong spatiotemporal variability in physical and biotic factors that can influence both spat set and the health of adults (Lenihan and Peterson 1998). For systems with limited water exchange and/or small tidal creeks with relatively large tidal ranges (> 1–2 m), minimum reef area may be essential for maintenance of local populations. In more open systems, increased cover may provide a buffer against local disturbances and recruitment variability.

IMPORTANCE OF HARVEST REFUGES

Unharvested (refuge) areas are critical to achieving both harvest and ecological roles of oyster reefs. Refuge areas protect brood stock and, as a result, can enhance oyster populations in surrounding harvested areas that are many times the size of the refuge itself (Wesson 1998). Moreover, in areas affected by oyster diseases, refuges provide protection for individuals that may have some resistance to disease. In harvested areas, the largest oysters, which are the individuals that have survived in the presence of disease pressure and have the highest fecundity, are the ones culled from the population (Rothschild et al. 1994, Coen et al.1999b). Protecting some reefs from harvest should, therefore, serve to enhance the vigor of stocks.

In addition, harvest-free sanctuaries allow reefs to develop and retain vertical relief and structural complexity that are important to both oysters and associated fauna. Vertical relief can provide oysters with the means to avoid near-bottom oxygen depletion and high sedimentation rates, and to take advantage of increased flow velocity and enhanced growth rates (Lenihan et al. 1996, Lenihan and Peterson 1998, Lenihan et al. 1999). In addition to reef elevation, vertical complexity of the reef itself (i.e., the presence of high culms interspersed with low areas) enhances fish and decapod utilization (e.g., Breitburg et al. 1995, Breitburg 1999, Coen et al. 1999b, Harding and Mann 1999, Posey et al. 1999, Coen and Luckenbach 2000) and may protect oyster spat from predation (Wesson 1998, unpubl. data, Giotta and Coen 1999). Because harvesting reduces vertical complexity, these habitat functions may benefit from creation of unharvested (refuge) areas (Coen et al. 1999b, Lenihan and Micheli 1999). However, there is also a view that some thinning may enhance intertidal oyster populations (Lenihan and Micheli 1999, W. Anderson, South Carolina Department of Natural Resources, pers. comm.).

Refuges also provide a tool at the landscape level that allows reefs to be placed in areas that are protected or closed to harvest and that will maximize desired functions (reviewed in Lenihan and Peterson 1998, Coen et al. 1999b, Luckenbach et al. 1999, Coen and Luckenbach 2000). For example, low-salinity refuge areas in the Maryland portion of the Chesapeake Bay are designated to protect oyster brood stock in areas generally unaffected by either *Perkinsus* (Dermo) or *Haplosporidium* (MSX) (Bushek and Allen 1996a,b, Paynter 1999, Coen and Luckenbach 2000). Similarly, designated areas closed to direct harvesting for health reasons may act as refugia as an indirect result of their value as habitat and brood stock reserves (Coen and Luckenbach 1999).

SPATIAL CONSIDERATIONS

There is still much to be learned about the importance of the location of restored oyster reefs within an estuarine landscape (Posey et al. 1998, Coen et al. 1999b). Whitlach and Osman (1999) have developed a metapopulation demographic model of oyster populations that illustrates the importance of dispersal between spatially distinct subpopulations to the persistence of oyster reefs. The foregoing discussion about brood stock sanctuaries and the dispersal of larvae from them to nearby reefs clearly illustrates the importance of reef position within a landscape to the development of reefs and potential fisheries production. Further, the location of reefs will affect the ecosystem services that they provide (see Lenihan and Peterson 1998, Coen et al. 1999b, Coen and Luckenbach 2000). For instance, restoring or constructing reefs in locations key to intercepting waters with high nutrient loadings and the associated high phytoplankton biomass should be possible. Similarly, the proximity to other structured habitat may be important to the function of oyster reefs (Micheli 1997, Coen et al. 1999b). Reefs could be sited in areas with little or no other structured habitat so that they could function as important "stepping stones" or migration corridors along the landscape. Alternatively, if data indicate the advisability of doing so, reefs could be sited in close proximity to other structured habitat to maximize interactions and connections between, for example, submerged aquatic vegetation or salt marsh grass and oyster reef assemblages.

A particularly intriguing ecosystem service provided by constructed oyster reefs adjacent to salt marshes has been discussed by Meyer et al. (1996, Meyer et al. 1997). In addition to providing structured habitat for fauna, these reefs stabilize the creek banks and reduce erosion of adjacent marshes (Meyer et al. 1996, Mayer et al. 1997, Meyer and Townsend 2000). As more information is gathered, the role of oyster reefs in erosion control may be determined to be as important as their other ecological services. Reefs with substantial vertical relief that reach the surface of the water may dissipate much of the energy generated where fetch on open bodies of water allows substantial energy to accumulate.

Regardless of other spatial considerations for oyster reef restoration and creation, several aspects of the placement of reefs within the landscape will influence their success both in terms of reef longevity and their measurable, short-term impact on the surrounding habitat. Successful siting of reefs generally depends upon locating substrate capable of supporting the added shell (without rapid burial), and therefore, generally favors their construction on footprints of historical oysters reefs. In addition, placement of brood stock sanctuaries should consider local circulation to maximize retention and recruitment of resultant larvae. This philosophy has dictated the placement of constructed reef sanctuaries in the Virginia portion of the Chesapeake predominantly in small subestuaries with limited watersheds, small tidal excursions, and basin topographies that encourage gyre-like circulation near the river mouths (Haven et al. 1981, Southworth and Mann 2000, Wesson unpubl. data).

STRATEGIES FOR RESTORATION

The harvest and ecological goals of oyster restoration are most likely to be compatible where management efforts focus on the ultimate goals, and the harvest is managed as a sustainable rather

than a "put-and-take" fishery. For example, targets for the amount of acreage for oyster restoration and protection could be set by determining the volume of water to be filtered within a given time or by determining the ratio of unharvested to harvested area required to sustain a target harvest quantity. We argue that such goal-oriented target setting is more likely to achieve the desired result than setting targets based upon historical oyster populations. Moreover, it is important to consider that restoration efforts proceed one step (i.e., one or a few reefs) at a time and that metrics to gauge the success of these efforts need to reflect both the value of the individual projects and their contribution toward the ultimate goal. For instance, the harvest potential of an individual reef expressed in terms of the biomass that may be harvested sustainably per unit area (rather than as the number of bushels of market-sized oysters in the standing stock) embodies both the productivity of the reef and the total area necessary to achieve the desired harvest levels. Similarly, the fishery value of a protected (unharvested) refuge area based on its potential contribution to harvest in other areas after allowing for a number of years of reef development is a more reasonable assessment of the value of a refuge than would be a simple calculation of the number of acres taken out of the active fishery. Likewise, measures of the ability of a unit area of reef to filter a specified volume of water or to support a specified biomass of finfish, decapods, shorebirds, or other target species will be more useful metrics than attempts to define the contribution of a single reef to the percent of the entire water mass filtered each day or to the biomass of a particular fish within an entire estuary.

LEARNING FROM RESTORATION EFFORTS

Restoration efforts, when properly designed and monitored, present an unparalleled opportunity to improve our understanding of both the optimal design for future restoration efforts and the ecological role of oyster reefs in coastal systems (Table 1). There are two key elements required to maximize the information from restoration efforts. The first is careful planning in the design and siting of reefs to match the restoration efforts with the information desired. For example, in areas such as the northern portion of the Chesapeake Bay and Delaware Bay where subtidal reefs were likely the historical norm, there may be concern that reefs not visible from but near the surface of the water may present navigation hazards. However, constructing reefs in deep water (thus, creating no navigation hazard) can expose oysters and associated biota to low dissolved oxygen concentrations during summer. By constructing and monitoring replicated reefs similar in size and relief (and thus cost) at shallow and deep sites, the optimal depth for reef placement in future restoration efforts could be determined. Simultaneously, important basic information could be gathered on the similarities and differences in the oyster populations and the ecological functioning of deep and shallow oyster reefs. More generally, by designing restoration efforts to allow comparisons between reasonable alternatives, it becomes possible to answer many important restoration questions. These include such questions as: (1) Does the benefit (i.e., growth, recruitment, or survival of oysters) derived from the construction of high vertical relief beds outweigh the costs of constructing such reefs? (2) Do oyster reefs placed near other structured habitats (such as SAV beds or tidal marsh areas) have higher or lower habitat value for finfish? (3) Is the extended "footprint" (i.e., area of increased oyster recruitment surrounding restored reefs) greater near harvested or unharvested restoration sites? (4) Does the addition of juvenile or adult brood stock oysters (either wild or hatchery-reared) increase long-term productivity of a reef sufficiently to justify the costs? (5) Does the benefit of oysters' water filtration

TABLE 1.

Examples of restoration efforts.

Restoration Action	Improvement in Restoration Practices	Improvement in Understanding of Oyster Reef Function
1. Reefs constructed at different depths	Importance of reef depth to successful restoration	Relationship between depth and recruitment, growth and survival of oysters and reef associated biota
2. Reef construction using different base materials	Evaluation of alternative materials for successful restoration	Relationship between construction material and development of oyster populations and reef associated biota
3. Reef construction with varying spatial dispersion patterns	Aid in the placement and spatial arrangement of restored reefs	Evaluation of the role of reef spacing patterns in maximizing oyster recruitment and providing habitat for mobile species
4. Position constructed reefs in varying proximity to other landscape elements	Aid in the placement and spatial arrangement of restored reefs	Evaluation of the importance of reef placement within a landscape for achieving restoration goals
5. Reefs constructed in areas with different tidal ranges and water quality and harvesting status	Aid in the successful restoration and protection of habitats that might otherwise not be protected or restored successfully	Enhance appreciation of EFH or critical habitat roles; provide better understanding of biogeographic differences among sites differing in physical regimes
6. Reefs constructed with varying shapes and vertical structure	Aid in the placement and construction of restored reefs	Evaluation of reef morphology relationships for habitat goals

Restoration efforts can be designed in ways that will provide information critical for improving future restoration work. In addition, they provide the opportunity for large-scale ecosystem manipulations that may greatly improve the understanding of the structure and functioning of coastal systems. The examples of these opportunities in the table are intended to be illustrative, not exhaustive.

capabilities vary with location, depth, habitat type, shape, etc.? (6) How do the shape and vertical complexity of reefs affect habitat function? (7) How do the answers to these and other related questions differ among sites and systems (e.g., intertidal versus subtidal oyster reef habitats, areas with significantly different tidal ranges, etc.)?

The second element required to maximize information from restoration efforts is the necessity for adequate monitoring to evaluate their success (see discussions in Coen and Luckenbach 2000). The specific type and intensity of monitoring will be determined by the goals of any particular restoration effort, the comparisons being made (as above), the target levels being set for improved harvest and ecological benefits, and ultimately the available funding. In addition, evaluation of both the biological impact of reef restoration (both harvest and ecological benefit goals) and the economic considerations may often be important. Experiences from the past several decades with restoration of other marine and coastal habitats consistently point to the need for well-designed monitoring studies to evaluate the success of restoration efforts (see Thayer 1992). As pointed out by Zedler (1992), monitoring to assess success and research that can help clarify how to meet restoration goals, are often not supported adequately by the entities that fund the actual restoration projects (discussed also in Coen and Luckenbach 2000). A significant challenge for oyster reef restoration efforts will be developing potential funding sources to support both large-scale habitat manipulations and long-term monitoring and assessment activities.

By combining carefully planned and targeted restoration efforts with adequate monitoring of the results, it will be possible to obtain information on topics about which little is known. Some of these topics (see also Table 1) are: (1) the characteristics of oyster reefs that are important for transient finfish and crab populations; (2) the area beyond the boundaries of the actual restoration effort in which both oysters and associated biota are affected under a range of hydrographic conditions; (3) the importance of the spatial arrangement of reefs within an estuarine landscape; and (4) the potential for oyster reefs to play a role in reducing shoreline erosion. These are not simply topics of academic interest but relate to the core goal of restoring oyster harvests as a sustainable fishery and minimizing anthropogenic effects to our coastal systems. In addition, among the most critical issues for future restoration efforts may be the questions: Where can sufficient reef substrate be obtained? and What oyster strains should be used to restore areas where oysters have long been in decline? Alternative substrates take on an increasingly significant role, as does the potential problem of introducing nonindigenous species or new disease strains with the importation of oyster shell from other regions (Bushek and Allen 1996a, Busheck and Allen 1996b, Bushek 1997, Coen et al. unpublished, G. Ruiz pers. comm.).

FUTURE STEPS

Despite uncertainties surrounding many aspects of reef restoration, it is important to move forward with restoration efforts; it is clear that reef restoration has the potential to provide strong benefits to both the harvest and ecological functions of oyster reefs in coastal systems. Most important, restoration efforts should target all three functions of natural reefs: harvest, the provision of structured habitat, and the potential for improved water quality. Rather than an adversarial relationship between fisheries and conservation interests in this regard, we suggest there are enough similarities of interests and approaches—especially the desire to optimize the amount and location of settlement substrate—that compatible strategies may be achieved. A critical feature of achieving this compatibility will be clearly expressing the benefits of reef restoration (depicted in Fig. 1), and relating each benefit in a quantifiable way to reef and oyster production.

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USE OF OYSTER SHELL TO CREATE HABITAT FOR JUVENILE DUNGENESS CRAB IN WASHINGTON COASTAL ESTUARIES: STATUS AND PROSPECTS

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ABSTRACT The deployment of oyster shell in estuarine intertidal areas to create habitat for juvenile Dungeness crab (*Cancer magister*) is now used routinely as mitigation for "unavoidable losses" of crab during dredging operations in Grays Harbor and Willapa Bay along the southwest coast of Washington State. Feasibility studies were conducted in 1986 to 1987 for a U.S. Army Corps of Engineers project to widen and deepen the navigation channel in Grays Harbor. Since that time, several studies have elucidated the ecology of crab and other organisms that recruit to the created shell reefs. Studies have also refined the procedures used to calculate crab losses caused by dredging and crab production in the shell habitat. The shell does serve as crab habitat; however, initial assumptions about the longevity of the shell have proved to be overly optimistic, because the shell can sink or be covered with silt before the end of the first summer after deployment. In addition, competition with the shore crab, *Hemigrapsus oregonensis*, has displaced juvenile Dungeness crab. We summarize results of these studies and present initial results from an ongoing mitigation effort that seeks to produce a more persistent living oyster reef in Willapa Bay.

KEY WORDS: Dungeness crab, dredging, mitigation, oyster shell, Washington State

INTRODUCTION

A large project that widened and deepened the navigation channel of Grays Harbor estuary in Washington State, USA (Grays Harbor Navigation Improvement Project, GHNIP) continues to be the impetus for numerous studies on the potential effects of dredging on the environment (USACOE 1982, USACOE 1989). Early on, Dungeness crab (*Cancer magister*, Dana, 1852) was identified as one of the most important species impacted by dredging operations in Grays Harbor (Tegelberg and Arthur 1977, Stevens and Armstrong 1984), and numerous studies were conducted to define those impacts (Armstrong et al. 1987, McGraw et al. 1988, Wainwright et al. 1992).

State and federal agencies directed the U.S. Army Corps of Engineers (USACOE) to minimize the loss of Dungeness crab caused by dredging or to mitigate for unavoidable losses (USACOE 1989). Based on observations that shell deposits found in the intertidal areas of Grays Harbor serve as habitat for newly settled Dungeness crab (Armstrong and Gunderson 1985, Gunderson et al. 1990, Jamieson and Armstrong 1991, Eggleston and Armstrong 1995), an extensive pilot study was conducted to demonstrate that shell deposits could be artificially created by placing oyster shell on the mudflats (Dumbauld et al. 1993). The habitat created was expected to enhance intertidal recruitment and survival of juvenile Dungeness crab and provide an economical means of mitigating for the loss of older, subtidal crab attributable to dredging. The GHNIP was approved and dredging completed in 1990 by removing 10 million m^3 of sediment from the navigation channel in Grays Harbor. To the extent possible within the confines of an economical dredging program (McGraw et al. 1988), the USACOE scheduled dredging operations to coincide with low crab abundance. Nonetheless, impacts occurred, and the USACOE utilized a model to estimate the losses at 100,000 adult equivalents (Wainwright et al. 1992). This represented less than 1% of the estimated crab population, but this loss was significant enough that USACOE was obligated to mitigate for the loss by creating intertidal shell habitat. A series of large shell plots were created on the intertidal mudflats of Grays Harbor from 1992 to 1998 to mitigate for the loss of crab caused by the initial construction of the channel, annual incremental maintenance, and other subsequent dredging projects.

In this paper, we review a pilot study and associated research that led to the current policy of mitigating for the loss of Dungeness crab by creating intertidal shell habitat in Washington State coastal estuaries. Problems are described that continue to be experienced in the implementation of the policy by constructing full-scale shell reefs, and potential solutions that have been developed based on continued research are summarized. We also introduce a more recent project that seeks to mitigate for the loss of crab attributable to a test dredging project in Willapa Bay, Washington. A significant difference in the mitigation strategy for the latter project is the attempt to take advantage of natural oyster recruitment occurring in this estuary. This is designed to create a living oyster reef much like the reefs being restored on the East Coast of North America (discussed elsewhere in this symposium proceedings). Our intent in this paper is to describe a novel approach to creating habitat for a decapod crustacean on the West Coast of North America, and also to highlight the perils and practical les-

sons learned from implementing such large-scale restoration efforts.

PILOT STUDY

Early observations indicated juvenile Dungeness crab recruit to and survive in shell deposits (typically death assemblages from the eastern softshell clam *Mya arenaria* L., 1758) and in aquaculture areas where Pacific oysters (*Crassostrea gigas*, Thunberg, 1793) are raised for human consumption (Armstrong and Gunderson 1985, Gunderson et al. 1990, Jamieson and Armstrong 1991). We conducted a pilot study to test the hypothesis that additional shell placed in the intertidal area of Grays Harbor, Washington, would serve as a refuge for and increase survival of juvenile crab, thereby mitigating for substantial losses attributable to subtidal dredging during construction of the GHNIP (Dumbauld et al. 1993). Oyster shell was placed intertidally at three locations in Grays Harbor (Fig. 1, small plots). At each location, three 15 × 15 m plots were constructed, and treatments were: (1) heavy shell cover (2–3 shell layers thick; (2) light shell cover (one shell layer thick); and (3) shell piles (approximately 1 m in diameter and 0.5 m high). Crab recruited to the shell plots from May to July and by late August grew to fourth–sixth instars (J4–J6, 17–31 mm carapace width, CW), then presumably emigrated to the subtidal. Results indicated that location within the estuary influenced crab abundance and that the three-dimensional configuration of the shell was also important (Fig. 2). Although there was statistical interaction between the location and treatment terms, when low numbers and variable results from the South Bay were removed from the analysis, it was determined that crab density was significantly higher as three-dimensional habitat increased in the sequence: shell piles > heavy shell cover > light shell cover. Subsequent research has shown that the shell habitat provides small crabs refuge from predation and that the thick shell treatment also provides better habitat than eelgrass (Fernandez et al. 1993).

The integrity of the shell habitat was measured 1 and 2 years after shell placement. The heavy shell treatment seemed to survive winter storms and sedimentation better than the piles treatment and crab continued to use the plots in subsequent years, albeit in lower densities. Because the heavy treatment was also the most practical to implement on a large scale, USACOE chose this configuration as part of the preferred alternative in the supplemental Environmental Impact Statement (EIS) (USACOE 1989).

LARGE-SCALE IMPLEMENTATION: PROBLEMS AND LESSONS

Shell Retention

The GHNIP received approval from state and federal agencies and dredging removed 10 million m^3 of sediment from the navigation channel of Grays Harbor in 1990. Results of the pilot study were used to plan for shell mitigation on a large scale. Recognizing the importance of site selection, the USACOE conducted studies in 1990 to 1991 (Armstrong et al. 1992). Three of four 0.4-ha plots

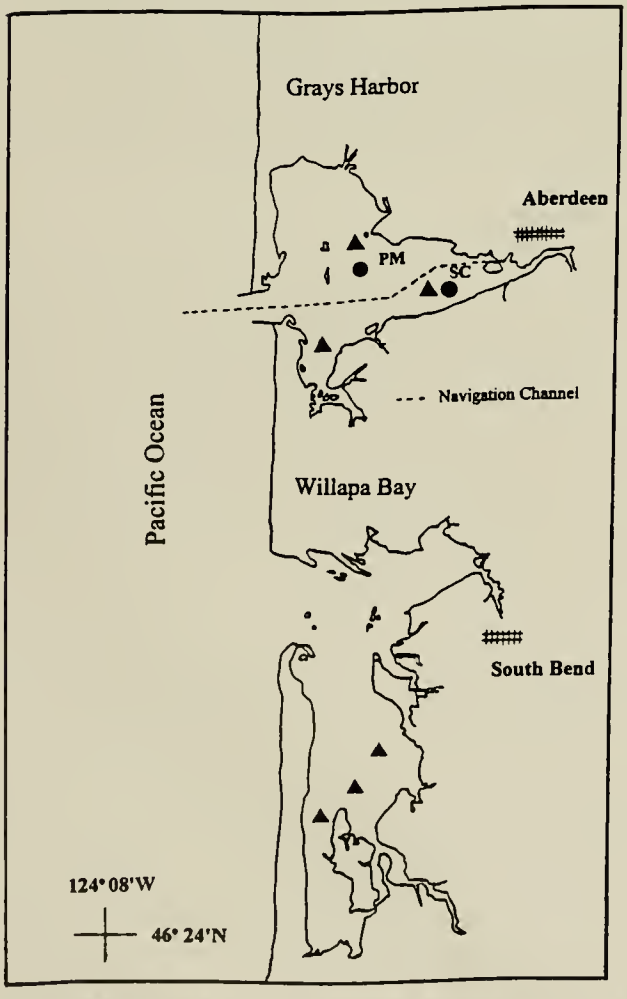

Figure 1. Map of Grays Harbor and Willapa Bay, Washington, showing locations of large-scale shell mitigation plots at Pacman (PC) and South Channel (SC) in Grays Harbor (●) and three smaller plots in Willapa Bay (▲).

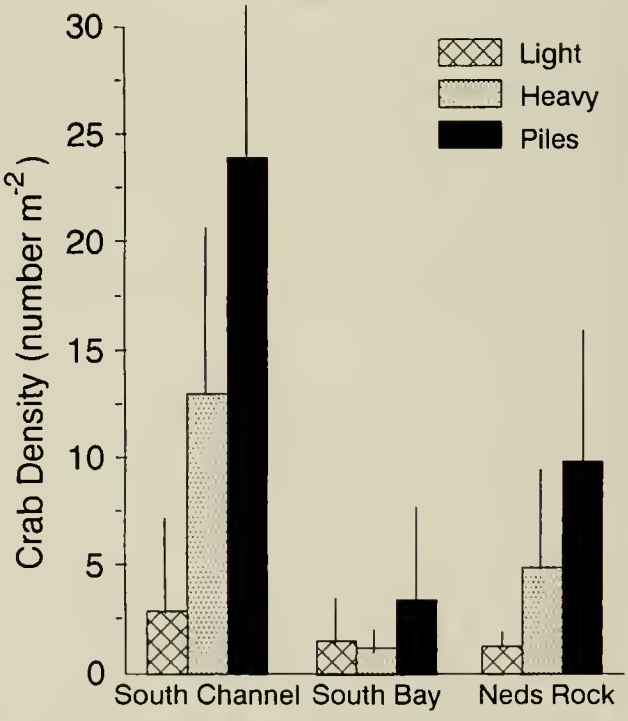

Figure 2. Time averaged (May–September) 0+ crab density (± 1 SD) in light, heavy, and shell pile habitat configurations created at three locations in Grays Harbor estuary during a 1986 pilot study (adapted from Dumbauld et al. 1993).

constructed in 1990 disappeared because of shell sinking into the substrate or sedimentation, so, in a second experiment conducted in 1991, test plots (30 m × 30 m) were placed at eight intertidal locations distributed throughout the Grays Harbor estuary. Shell sank below the surface at the majority of these sites within the first 2 weeks after construction. Tests, using a very sensitive surveyor's level to determine relative elevations and produce contour maps, showed that the process most responsible was subsidence attributable to bioturbation by resident thalassinid shrimp (Fig. 3). Sedimentation occurred as well (i.e., shell remained above grade level but was covered by additional sediment), but this process seemed to be correlated with shrimp density.

Full-scale mitigation began in 1992 in Grays Harbor with construction of two large shell plots 6.7 ha and 2.2 ha at locations known as South Channel and Pacman respectively, (Fig. 1). Shell retention by August of that year was only 32% at Pacman because of relatively high shrimp density (60 burrows/m^2), and because the shrimp present were ghost shrimp (*Neotrypaea californiensis*, Dana, 1854), which cause more sedimentation than mud shrimp (*Upogebia pugettensis*, Dana, 1852) (Dumbauld et al. 1996, Dumbauld et al. 1997). Shell retention was about 70% at South Channel, where a mix of mud shrimp and ghost shrimp were present but at much lower density (20 burrows/m^2). Although remaining shell at both sites produced juvenile crab, all subsequent mitigation projects in Grays Harbor have been at the South Channel location because of better shell retention. Based on pilot study results, the USACOE predicted that shell retention would level off at 50–80% after the first 2 years (USACOE 1989); this was not realized in the full-scale mitigation project (Dinnel 1996, Fig. 4). An exponential curve fits the data, but the slope of the retention curve is site dependent. Similar studies in Willapa Bay have since linked shell subsidence directly to abundance of shrimp and the species of shrimp present (Feldman et al. in press, Fig. 4). Finally, USACOE hoped that harrowing the shell as practiced by the commercial oyster industry (Sayce and Larson 1966), might return shell to the surface and provide for additional shell life and, therefore, crab recruitment. This was to be carried out in year 4 and was projected to return surface cover to 90% of the original. Initial tests, although never quantified, indicated that harrowing would not succeed.

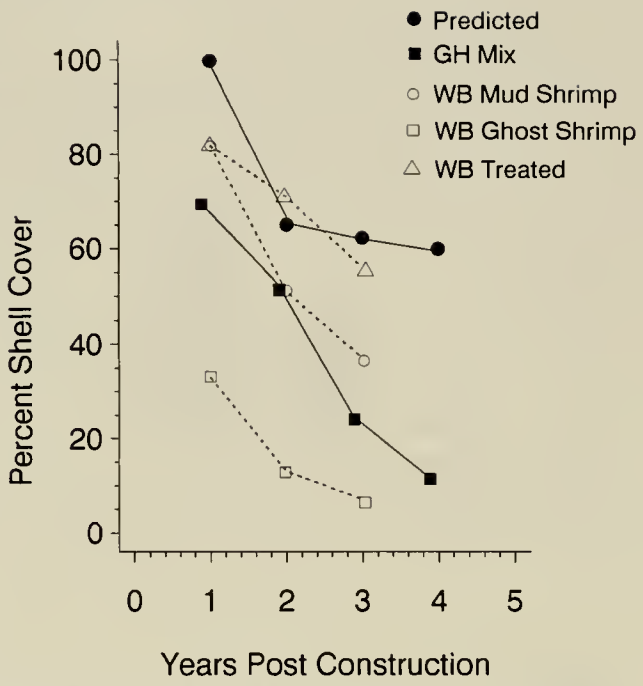

Figure 4. Shell retention over time as predicted by USACOE (●) and realized at the plots constructed in the South Channel area of Grays Harbor in 1992 (■). Both ghost and mud shrimp were present at this site. Also shown are similar retention curves for Willapa Bay at a site with ghost shrimp present (□), a site where only mud shrimp were present (○), and a site that had ghost shrimp present, but was pretreated with the pesticide carbaryl to control the shrimp (△).

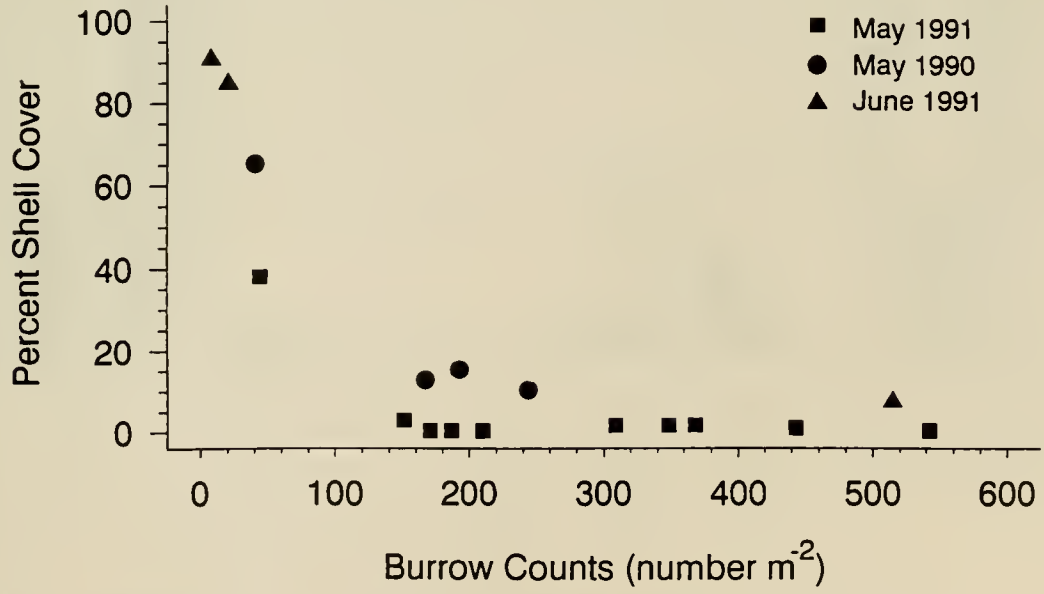

Figure 3. Relationship between shrimp burrow counts and percentage shell cover 1–2 months after deployment at 14 sites in Grays Harbor estuary. Note the significant negative correlation ($r = -0.68$, $P < 0.05$) and approximate threshold near a count of 100 burrows/m^2 above which shell retention is less than 10% (after Armstrong et al. 1992).

Faunal Composition

As part of a monitoring plan, composition, density, and/or biomass of several taxa were measured in the created shell habitat and, in some cases, compared with fauna in adjacent open mudflats. In addition to recruitment and density of juvenile Dungeness crab, two issues of interest were: (1) amount and composition of other fauna that may serve as food for Dungeness crab; and (2) the recruitment and spatial distribution of both species of thalassinid burrowing shrimp.

Certain fishes (e.g., the saddleback gunnel, *Pholis ornata*, Girard, 1854) and small crustaceans (mostly amphipods such as *Eogammarus confervicolus*, Bousfield, 1979, and *Corophium spp.*) in addition to Dungeness crab had much higher biomass (and density) in shell as compared to open mud (Williams 1994, Fig. 5). We found a relationship between the depth of shell and relative density and biomass of gammarid amphipods (up to 6,000 individuals or 4g/m^2 ash free dry weight, AFDW), but no relationship with juvenile Dungeness crab density (Fig. 6). Several important prey taxa, including juvenile bivalves and polychaetes, occurred at comparable biomass (0.7 and 0.5 g AFDW/m^2, respectively) in shell and mud (Fig. 5). The quantities of small prey suggested the resident juvenile Dungeness crab population would have sufficient food to grow and develop to an instar size (J4–J5), at which size, they emigrate from the intertidal to subtidal environment (Visser 1997). Further research indicated that predation on juvenile clams (*Macoma balthica*, L., 1758) increased in the shell habitat because of enhanced Dungeness crab abundance, holding the standing stock of clams to a similar level as that found in the open mud (Iribarne et al. 1994, Iribarne et al. 1995).

Recruitment and distribution of 0+ (young-of-the-year) thalassinid shrimp differed across the two dominant habitats in accord with species. Related to the use of shell as habitat for juvenile crab was whether shell would also reduce burrowing shrimp density and have potential as an alternative to using carbaryl to control these species that have a negative impact on oyster culture operations (Dumbauld et al. 1996, Dumbauld et al. 1997). Very high densities of 0+ ghost shrimp were found in adjacent

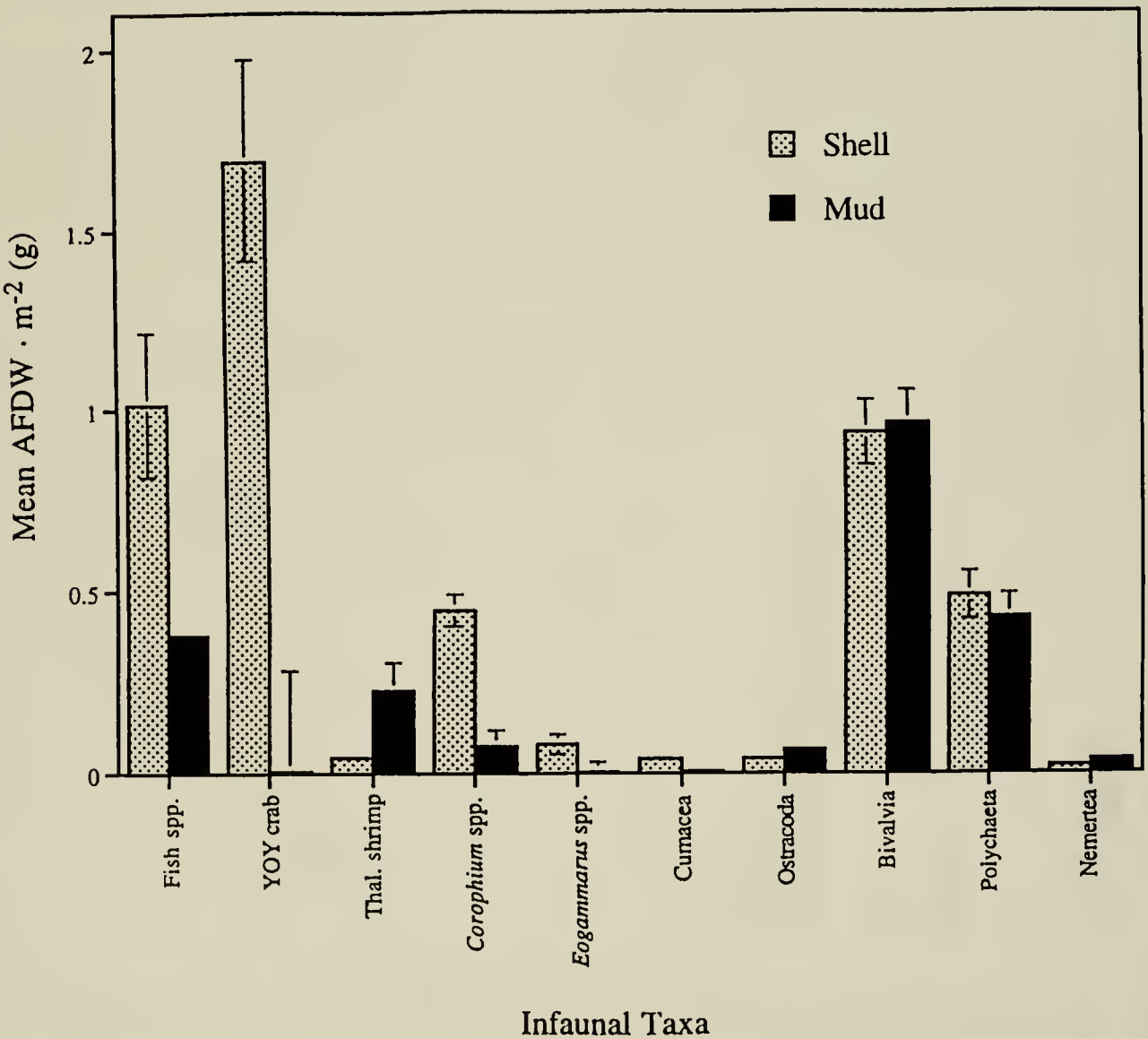

Figure 5. Mean total biomass (ash-free dry weight, AFDW)* 1.0/m^2 (±1 SE) of major infaunal taxa from shell and open mud control plots in Grays Harbor pooled across locations and months. Crab biomass estimates are based on values for J2–J3 instars (after Williams 1994).

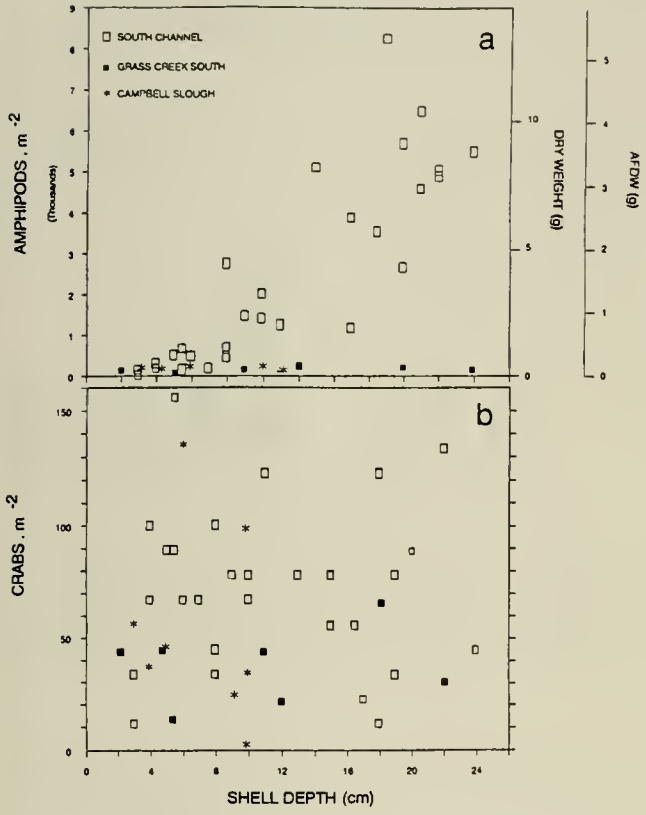

Figure 6. Amphipod and crab densities in shell samples from August 1990. A significant relationship between amphipod (*Eogammarus confervicolus*) density and shell depth is evident (A) whereas, there is no relationship between Dungeness crab density and shell depth (B). Also given is a scale for biomass (AFDW, g) for amphipods (after Armstrong et al. 1992).

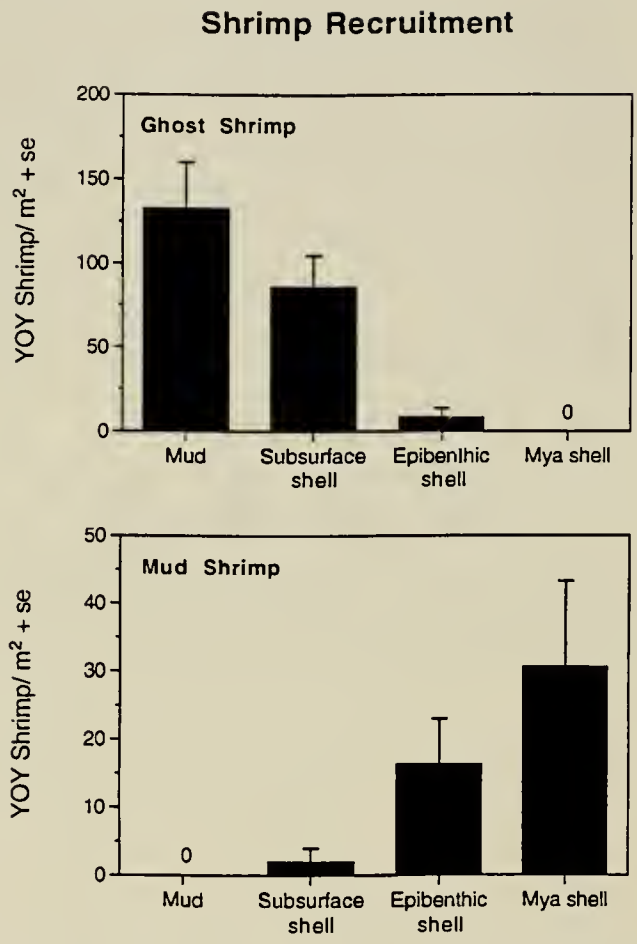

Figure 7. Number of young-of-the-year (YOY) thalassinid shrimp recruiting to open mud and various categories of shell cover, including shell that had already been buried below the surface (subsurface shell), a thick layer of oyster shell (epibenthic shell), and deposits of eastern softshell clam shells. Ghost shrimp (top) were most abundant in open mud and sand, and mud shrimp (bottom) were most abundant in shell deposits, reflecting both settlement behavior as well as timing relative to predation pressure by resident YOY crab.

open mudflat habitat and in mud overlying shell that had sunk below the surface (Feldman et al. 1997). Conversely, high densities of 0+ mud shrimp were found in naturally occurring *Mya* shell deposits and in oyster shell mitigation plots (Feldman et al. unpubl. data, Fig. 7). Feldman et al. (in press) concluded that shell generally reduces population density of juvenile ghost shrimp because of its function as a physical barrier and settlement deterrent and/or as a refuge for high densities of 0+ Dungeness crab, a predator. Mud shrimp seem less affected by surface cover of heavy shell, and, although their burrowing activity is less than that of ghost shrimp, they could pose a greater long-term threat to shell integrity.

Competition

During 1992, the initial year of shell plot construction, the abundance of Dungeness crabs that recruited to the plots was high, but few other crabs were seen. However, in subsequent years, the abundance of yellow shore crabs (*Hemigrapsus oregonensis*, Dana, 1851) found in the shell plots increased dramatically, and a negative correlation was noted between the density of these two species (Fig. 8). Further detailed investigations indicated shore crabs recruited as juveniles to the plots via larval settlement and also as adults by moving from adjacent areas (Visser 1997). Visser found the mechanism responsible for the inverse relationship between the two species of crab to be direct competition for space and not predation. Shore crabs are predominantly herbivores and scavengers, although they do consume some Dungeness megalopae. Shore crabs are very active competitors for refuge space and won an average of 78% of interspecific encounters when they were of comparable size to the Dungeness crab (Visser 1997). Because Dungeness crabs recruit to the shell on an annual basis and emigrate later at larger size to the subtidal, they are at a size disadvantage when they settle in areas where shore crabs have had time to colonize, because the latter are year-round residents. Visser (1997) also found that Dungeness crab megalopae tend to avoid settling in areas where shore crabs are present. This result was entirely unexpected and forced USACOE to reconsider the strategy of obtaining multiyear crab recruitment from shell plots that were more than 1 year old. Instead, they had to rely on new habitat construction every year.

Interannual Variability in Settlement

Dungeness crab display cyclic abundance, in part because of their pelagic larval life history, which results in variable recruitment to the benthos (McConnaughey et al. 1992, McConnaughey 1994) found a 40-fold variation in interannual settlement along the

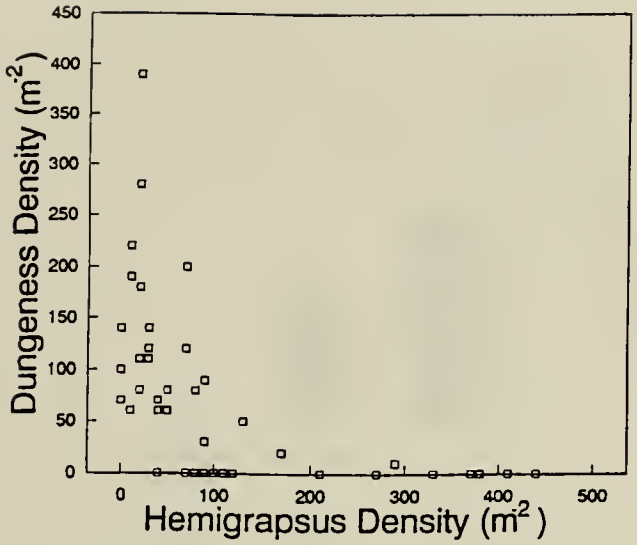

Figure 8. Density of 0+ Dungeness crab in shell mitigation plots at the South Channel location in Grays Harbor as a function of total density of shore crab (*Hemigrapsus oregonensis*) collected in the same samples from 1992 through 1994 (after Armstrong et al. 1992).

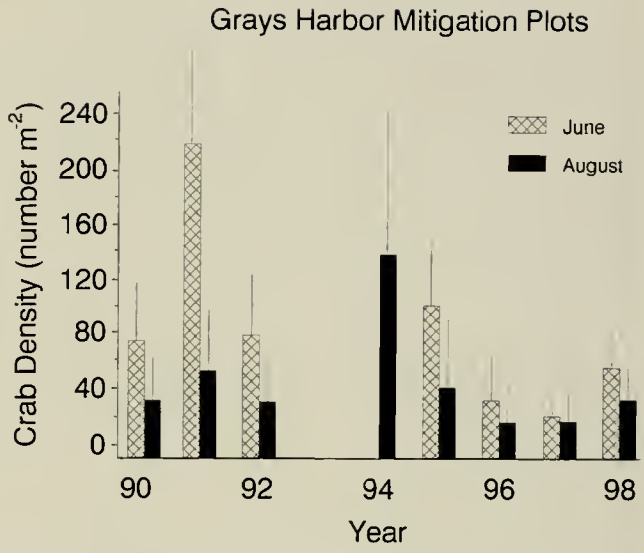

Figure 9. Average density of 0+ Dungeness crab (± 1 SD) collected in shell mitigation plots at the South Channel location in Grays Harbor from 1990 through 1998. Data were collected in 1990 to 1995 by the University of Washington and in 1996 to 1998 by Grays Harbor Community College.

Washington coast that was inversely related to the strength of alongshore and cross-shelf transport during the previous 4–5 month larval period. Crab settlement is also variable from location to location within the estuary because of wind-driven surface currents. However, Eggleston and Armstrong (1995) found that post-settlement mortality was more influential than larval supply in controlling the density of J1 instars at two locations in Grays Harbor. Density at settlement has varied substantially in the GHNIP constructed shell mitigation plot and to various other projects that have occurred since 1992 in the South Channel area of Grays Harbor; however, the average crab density in newly created shell in August was not significantly different between years (Fig. 9). Based on the results of the pilot study, USACOE chose a conservative average of 10 crab/m^2 (August) to estimate crab production for shell mitigation. This average has been met in all years, including 1992, the first year of habitat construction.

Crab Production and Dredge Entrainment Models

An adult equivalent loss model was developed to determine the number of crabs killed by dredging in the GHNIP (Wainwright et al. 1992). The model is based on a dredge entrainment function that relates the number of crabs entrained (estimated directly based on counts taken from a modified dredge) and the number of crabs present (estimated from trawls conducted simultaneously). Natural mortality estimates are used to convert different age classes of crab into adult equivalents. The model also incorporated seasonal and spatial information on crab density and size composition from the Grays Harbor estuary and USACOE dredging scenarios of gear type and amount of sediment dredged to project the number of crabs entrained and killed. The model produced an estimate of 162,000 age 2+ crab (near maturity) killed during GHNIP construction, which equates to about 73,000 3+ crab (age of recruitment to the fishery). The same mortality estimates were used to calculate the number of 0+ crabs necessary to mitigate for the loss, which came to about 9,500,000 crabs.

The original approach that USACOE took for estimating the number of 0+ crab produced by shell mitigation was based on the simplistic view that when crabs emigrate from the plots in July they are considered "produced." The standing stock density in August was multiplied by an estimate of the amount of shell remaining (total area multiplied by percentage shell cover) to give a production figure. This approach was later modified, and a production model developed that takes into account time, molt interval, growth, mortality, and multiple cohorts of settling crab (Armstrong et al. 1995). The model uses J2 density as a starting point and estimates mortality and growth from intertidal shell plot data. Results suggested either a J3 or J4 (last instar that inhabits the shell before emigrating) production unit could be valid, and J4 was selected by agency and USACOE biologists. USACOE recently refined all previously calculated mitigation estimates using this production model, and, in most cases, a higher estimate was obtained than that of the August standing stock method. This is thought to be primarily because of emigration, which begins before the August sampling date. The production model was updated and improved again based on 1998 field data showing initial settlement densities were extremely high, but survival rates were rather low. Additions to the production value were also made for crabs that were larger than the J2 starting point at the initial May sampling date. This accounts for settlement that can occur (to a minor extent) as early as March to April in Grays Harbor. Thus, the production model takes mortality rates for J2–J5 crab for each shell plot sampled and applies them to measured field density of each instar over the average time that instar spends at size before molting. This value is multiplied by the total habitat available for each month and summed to give the total number of 0+ crabs produced per plot.

SOLUTIONS AND PROSPECTS

Since 1995, an interagency work group has met to evaluate and update the USACOE crab mitigation program, and most recently, the agencies signed a strategy agreement (USACOE et al. 1998). The revised crab mitigation strategy is intended to update the Dungeness crab avoidance and mitigation measures outlined in the

original Environmental Impact Statement written for the GHNIP (USACOE 1982) in order to keep the program relevant to USACOE, the project sponsor, and agency concerns. It addresses a number of the factors listed above, including:

1. a renewed focus on and credits for avoidance of impacts, including reduced over-all dredging, a re-examination of clamshell dredging, and timing of downstream dredging to avoid peak months of crab abundance;
2. a commitment to shell mitigation for impacts remaining from past dredging and for any future impacts;
3. use of the production model (Armstrong et al. 1995) to calculate the number of young-of-the-year crab "produced" by the shell mitigation plots; and
4. continued efforts to promote impact avoidance to re-evaluate crab mitigation efforts, including semiannual meetings of the crab mitigation workgroup.

Finally, we introduce a project that attempts to mitigate for the loss of crab resulting from a test dredging project in Willapa Bay, Washington (Dumbauld and Kauffman 1999). This project represents a new twist in the mitigation strategy, because it attempts to create a living oyster reef as habitat for juvenile crabs. USACOE conducted test channel dredging on the sand bar at the mouth of Willapa Bay in August 1997 to determine the effectiveness of a new tool (agitation dredging with sidecasting) in this environment. A fairly small amount of material was removed (61,164 m^3). Bottom trawls for crabs were conducted, and USACOE agreed to mitigate for the loss of 1,931 crabs (age 2+). Given the potential for future mitigation projects in Willapa Bay, the crab workgroup used this mitigation project to determine whether techniques applied in Grays Harbor would work equally well in Willapa Bay. A second and perhaps more relevant goal for this work group was to investigate whether living oyster reefs could be created, because oysters spawn and set naturally in this estuary. The shell mitigation project was conducted in the Washington State oyster reserves in Willapa Bay. The reserves offered several advantages, including: (1) the Washington Department of Fish and Wildlife manages the reserves to protect and preserve oyster resources and, therefore, they offered an optimal site for examining creation of living oyster reefs; (2) the reserves are located in areas that typically receive larval oyster set; and (3) because oyster production is an existing activity on the reserves, permitting was simplified.

Approximately 2,567 bushels (90 m^3) of oyster shell were placed at each of three separate intertidal locations on the reserves to examine both crab and oyster recruitment in 1998 (Fig. 1). Shell placement sites were carefully selected to avoid areas with high thalassinid shrimp density; shell retention was approximately 65% in the first year. Crabs recruited to all three shell plots, but numbers were higher on the two plots located closer to the mouth of the estuary. Using the production model (Armstrong et al 1995), combined recruitment to all three plots was calculated to be about 147,000 J4 crab, which satisfied the mitigation requirement. Oysters recruited to the shell plots in late summer 1998 and began to form visible live oyster reefs in 1999, particularly at the station closest to the estuary mouth, where they were more abundant and grew faster. Crab recruitment was also highest at this location in 1999, but was markedly lower than the previous year. We hope to continue to monitor these plots to assess the value of the habitat provided by living oyster reefs as the oysters mature.

This is a brief overview of what has been and continues to be an interesting, but sometimes arduous process to mitigate for lost resources. One additional factor, not mentioned so far, is cost. Dinnel (1996) calculated that the cost of placing shell has increased since the inception of the project to the point where each crab, worth approximately $3 to the fisher, now costs USACOE at least $70 to replace through shell mitigation. With some of the refinements noted above, he notes this might be reduced by 50–60%; however, it is clear that avoiding impacts in the first place is still the most cost-effective tool. To the extent possible, USACOE and the other agencies involved have acknowledged this in the latest mitigation strategy (USACOE et al. 1998) by embracing creative steps to avoid crab loss as well as refining models to estimate crab production within shell. The cost–benefit ratio may be enhanced even further should it be possible to create living shell reefs that function as crab habitat beyond the initial year of construction as well as providing other ecosystem services.

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TOWARD DESIGN CRITERIA IN CONSTRUCTED OYSTER REEFS: OYSTER RECRUITMENT AS A FUNCTION OF SUBSTRATE TYPE AND TIDAL HEIGHT

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ABSTRACT Restoration of degraded oyster reef habitat generally begins with the addition of substrate that serves as a reef base and site for oyster spat attachment. Remarkably, little is known about how substrate type and reef morphology affect the development of oyster populations on restored reefs. Three-dimensional, intertidal reefs were constructed near Fisherman's Island, Virginia: two reefs in 1995 using surfclam (*Spisula solidissima*) shell and six reefs in 1996 using surfclam shell, oyster shell, and stabilized coal ash. We have monitored oyster recruitment and growth quarterly at three tidal heights (intertidal, mean low water, and subtidal) on each reef type since their construction. Oyster recruitment in 1995 exceeded that observed in the two subsequent years. High initial densities on the 1995 reefs decreased and stabilized at a mean of 418 oyster/m^2. Oyster settlement occurred on all reef types and tidal heights in 1996; however, postsettlement mortality on the surfclam shell and coal ash reefs exceeded that on the oyster shell reefs, which remained relatively constant throughout the year (mean = 935 oysters/m^2). Field observations suggest that predation accounts for most of the observed mortality and that the clam shell and coal ash reefs, which have little interstitial space, suffer greater predation. Oyster abundance was consistently greatest higher in the intertidal zone on all reefs in each year studied. The patterns observed here lead to the preliminary conclusion that the provision of spatial refugia (both intertidal and interstitial) from predation is an essential feature of successful oyster reef restoration in this region. In addition, high levels of recruitment can provide a numerical refuge, whereby the oysters themselves will provide structure and increase the probability of an oyster population establishing successfully on the reef.

KEY WORDS: oyster, *Crassostrea virginica*, habitat restoration, recruitment substrate, intertidal, Virginia

INTRODUCTION

The marked decline in oyster resources in the mid-Atlantic region throughout much of this century have been attributed primarily to increased harvest pressure, a direct consequence of ineffective resource management (Haven et al. 1978, Rothschild et al. 1994, Frankenberg 1995). Furthermore, the increased prevalence of the protistan parasites *Perkinsus marinus* ("Dermo") and *Haplosporidium nelsoni* ("MSX") (Burreson and Ragone 1996) and over-all environmental degradation have accelerated declines in oyster numbers over the last three decades. There is a general consensus that oyster reefs were once a dominant feature of much of the lower Chesapeake Bay, contributing considerable biological and geological structure to the system. Historically, oysters in this system likely affected systemwide trophic structure and water quality (Newell 1988, Ulanowiz and Tuttle 1992), while providing considerable physical structure, which, in turn, facilitated the development of diverse benthic communities. The need to restore oyster resources and oyster reefs, not only for their direct harvest but also for the ecological services they provide, has been recognized recently (Lenihan 1996, Coen and Luckenbach in press, Coen et al. 1999).

To date, efforts to restore the resource have been focused in areas where the oysters were abundant and extensive but have been reduced to subtidal "footprints" of former reefs. Restoration attempts carried out in areas previously devoid of oysters (as described herein) have been few. Typically, restoration of a degraded oyster reef has involved the addition of substrate to serve as a reef base and site for spat attachment and subsequent oyster growth. Oyster shell resources and/or the funds to purchase them are often in limited supply; therefore, the interest in evaluating both how to use oyster shell most effectively and the efficacy of using alternative substrates as reef bases is considerable. Attention has recently been given to the importance of vertical relief of reefs on oyster growth, survival, and disease dynamics (Bartol and Mann in press; Lenihan et al. 1996, Lenihan and Peterson 1998); however, there remains a paucity of information on the degree of relief necessary to maximize oyster settlement, recruitment, and subsequent survival. Furthermore, numerous studies have investigated the use of alternative substrates to oyster shell (Soniat et al. 1991, Haywood and Soniat 1992, Haywood et al. in press). These studies have generally been laboratory or small-scale field experiments and have not clarified how these substrates might be used to maximize oyster recruitment, growth, and survival in the context of large-scale reef restoration. These issues have increasing relevance as restoration efforts proceed throughout the extensive range of the eastern oyster. This report focuses on a large-scale field experiment in the lower Chesapeake Bay, Virginia, which related oyster recruitment, growth, and survival to reef substrate types and tidal height. The results have relevance for the choice and placement of materials and the development of design criteria for oyster reef restoration.

SITE DESCRIPTION

The study site is located near Fisherman's Island, Virginia, U.S.A., in the vicinity of the mouth of Chesapeake Bay (Fig. 1). This is a polyhaline site with a tidal amplitude of approximately 1.25 m. Marsh islands, intertidal flats, and subtidal bottom within the area are all owned by the Commonwealth of Virginia and the

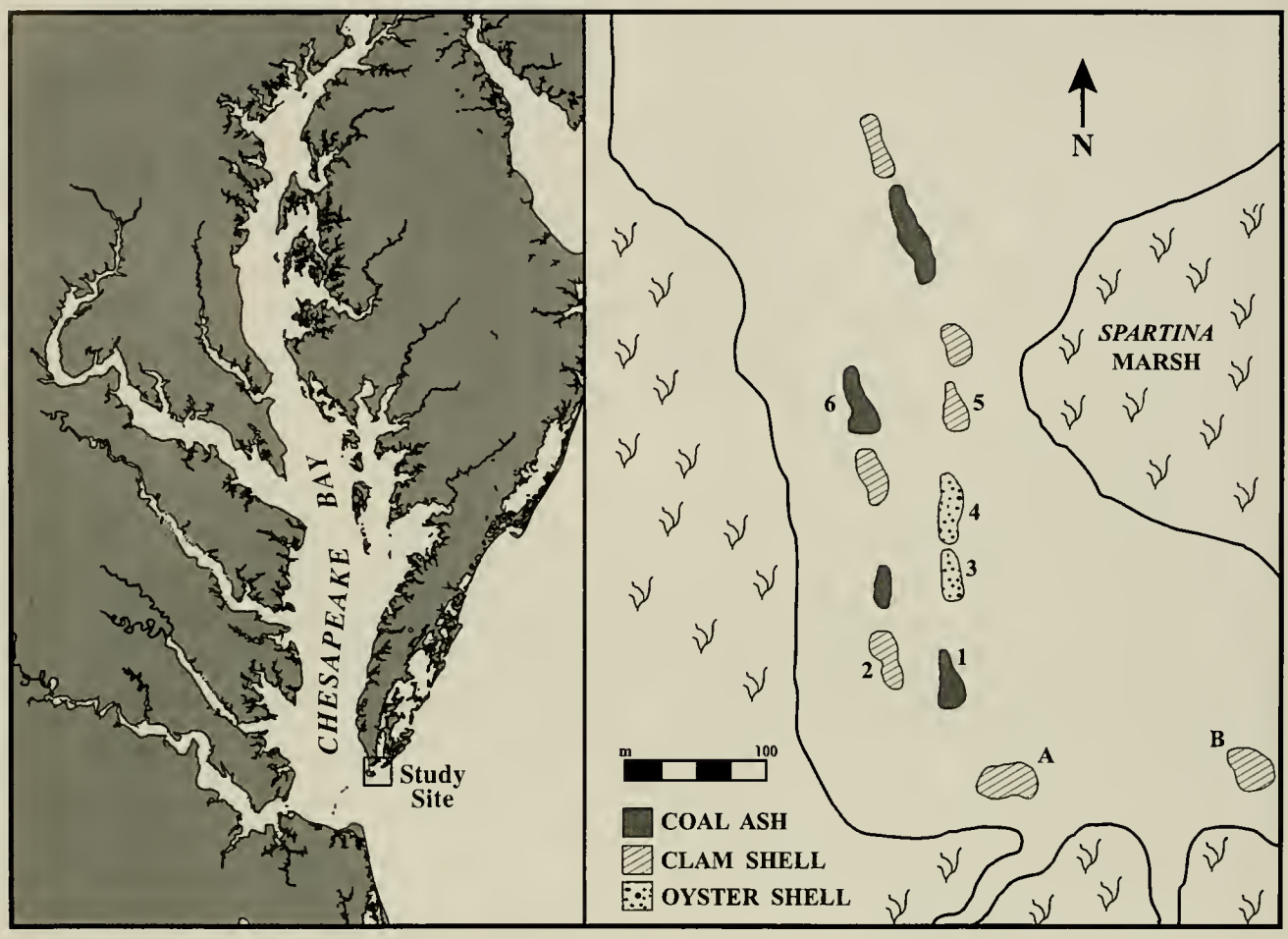

Figure 1. Location of study area near Fisherman's Island, Virginia. Reefs with an alphanumeric label were monitored continually throughout the period of the study. Reefs are not drawn to scale.

federal government and are managed by the U.S. Fish and Wildlife Service as part of the Eastern Shore of Virginia National Wildlife Refuge. In April 1995, two intertidal reefs, approximately 8,000 m^2 (2 acres) each, were constructed at the site as part of a remediation project funded by the Chesapeake Bay Bridge Tunnel District. The reefs were created by placing approximately 40,000 Virginia bushels (~ 1,973 m^3) of surfclam (*Spisula solidissima*) shells on two intertidal mudflats (see A and B in Fig. 1). The reefs extended from ~ 0.5 m below to 0.5 m above MLW. The reef designated A in Figure 1 had greater surface area at higher tidal elevation than reef B. Irregular patterns of mounds, ridges, and furrows existed across the reef surface as a result of the planting technique (deployment from barges by water cannon). Hereafter, the clam shell reefs, constructed in 1995, are designated as 95 Clam reefs.

Eleven additional reefs (Fig. 1) were constructed in 1996 with funding from the Aquatic Reef Habitat Program, Virginia Power Company, and the Virginia Oyster Repletion program. Five of these reefs were constructed with surfclam shells, two with oyster (*Crassostrea virginica*) shells, and four with stabilized coal combustion by-products (fly ash). The latter material, constructed using 88% fly ash stabilized with 12% (w:w) Portland cement, is described in greater detail in Andrews et al. (1997) and has been shown to provide an environmentally suitable substrate for oyster settlement and growth (Alden et al. 1996). Limited availability of oyster shells resulted in the smaller number of reefs (n = 2) constructed with that material. A total of 39,920 bushels (1,965 m^3) of surfclam shells, 7,000 bushels (325 m^3) of oyster shell, and 20,150 bushels (994 m^3) of coal-ash pellets were used to construct the reefs. Two reefs of each substrate type, ranging in size from 162 to 364 m^2, were selected for monitoring (reefs 1–6 in Fig. 1). The reefs were oriented in a north–south direction, with seven reefs in one row and four reefs in another row to the west. A channel ranging in width from 10 to 40 m separates the two rows. Hereafter, the reefs constructed in 1996 are designated as Oyster, 96 Clam, and Ash.

MATERIALS AND METHODS

Quadrate Sampling

Sampling of the reefs for determination of oyster abundance and size was initiated in October 1995. On each of the reefs selected for monitoring (two of each substrate type: A, B, and 1–6 in Fig. 1), quadrate samples (n = 3) were collected from each of three tidal heights. The tidal heights were 0.25 m below mean low water (hereafter called Subtidal), at mean low water (hereafter called MLW), and 0.25 m above MLW (hereafter called Intertidal). Replicate quadrates (0.0625 m^2; n = 3) were placed haphazardly within each tidal height stratum (Subtidal, MLW, and Intertidal) on replicate reefs (n = 2) of each reef substrate type

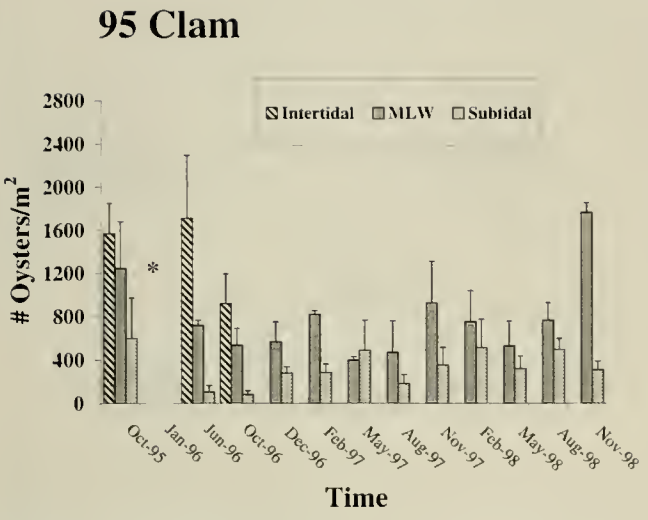

Figure. 2. Oyster abundance (number per m^2, mean ± SD) from three tidal heights throughout the study on the 95 Clam shell reefs. * No replicate quadrates were taken during this sampling period.

(Oyster, 95 Clam, 96 Clam, and Ash) to give a maximum of 72 samples per sampling period. Within each quadrate sample, all reef substrate was retained to a depth of 15 cm but did not include underlying sediments if encountered. Samples were transported to the laboratory on ice (if necessary) and were processed immediately. Processing involved the enumeration of all live oysters in each sample. In addition, 50 oysters from each tidal height, on each reef sampled, were measured to the nearest 0.1 mm. Sampling took place on a quarterly basis in an attempt to detect seasonal changes in oyster abundance.

Interstitial Space Estimates

The volume of interstitial space for each of the substrates used to construct reefs in 1996 was estimated using subsamples of the substrates before the deployment of the substrates. All of the subsamples used were considered the ideal for that substrate type: whole (with some partially fragmented) oyster and surfclam shells and ash pellets ≈ 5 cm in diameter. Interstitial volume was calculated using the volumetric displacement of the substrate packed to the top of a container (≈5.85 L). This displacement value was then subtracted from the container volume to give interstitial volume. All interstitial volumes were corrected to reflect the substrate type within a 1-L container. This process was carried out five times for each substrate in order to generate mean and standard deviation values. These values were then compared using a one-way analysis of variance ANOVA.

Statistical Analysis

The 95 clam reefs were not compared statistically with the 1996 reefs because of the dual confounding effects of temporal difference in deployment and considerable differences in surface areas of the reefs. Summary statistics generated for oyster densities and sizes by reef type are reported in graphical form.

Over the course of the study, some tidal height strata on some of the 1996 reefs were much reduced as a result of settling and/or erosion, thus we were unable to complete sampling from all tidal heights for the duration of the study. Therefore, for the purpose of comparing the abundance of oysters by substrate, we confined our analysis to the subtidal samples, for which there is a complete set of samples. Abundances were log transformed [ln (x+1)] to conform to normality assumptions as required. A two-way ANOVA was carried out (with substrate type and time as the main effects) to ensure that there was no interaction term. Upon satisfaction of this criterion, a randomized complete block design ANOVA was conducted using substrate type as the main effect blocked by time (Sokal and Rohlf 1981, pp. 345–352). The 96 Clam reefs had returns from all tidal heights for all time periods bar one (one reef in November 1997). Therefore, we were able to compare oyster abundances from all tidal heights of the 96 Clam reefs. The values from these tidal heights were compared accordingly. The Oyster reefs had complete returns from the MLW and subtidal samples for the two replicate reefs, resulting in valid comparisons of these tidal heights blocked according to time.

RESULTS

At the initial sampling of the 95 Clam shell reefs in October 1995, high oyster numbers were recorded at all tidal heights (Fig. 2). The intertidal samples had the highest oyster numbers throughout, followed by the MLW and subtidal samples, respectively. Subsequently, oyster abundances declined precipitously at all tidal heights. By November 1996, the elevations of the reefs were reduced through subsidence, compaction, and/or erosion to the point that intertidal samples could not be retrieved (Fig.2). Despite some fluctuations, the numbers of oysters on these reefs tended to remain stable in the following sampling periods. Throughout this period, the abundance of oysters remained fairly constant, mean values for the MLW and subtidal samples were 834 oysters/m^2 and 345 oysters/m^2, respectively. There were no appreciable differences in size distribution among the tidal heights through the sampling periods. Therefore, the size frequencies from each tidal height within each sampling period were pooled, and these are graphically represented in Figure 3. A unimodal population distribution is apparent for the first year of the monitoring (October 1995 to September 1996). Following a small recruitment event in December 1996 (Fig. 3), a bimodal population distribution was evident. Between August 1997 and November 1997, mortality among larger animals and an influx of small, newly recruited individuals was apparent. Thereafter, the size distribution on these reefs remained relatively stable, with small, newly recruited individuals dominating in terms of over-all abundance (Fig. 3).

Relatively low numbers of oysters were present in the Ash reef samples from December 1996 through August 1997 (Fig. 4). In November 1997, the young-of-the-year animals were detected on the reef and increased the over-all number of oysters sampled. The recruitment event in each year sampled was followed by a rapid decline in the numbers of oysters found on the reefs. Also, throughout the sampling of the Ash reefs, the intertidal stratum consistently contained higher oyster densities than the other tidal heights. The MLW stratum for the most part, had greater oyster densities than the subtidal stratum. The size distribution of oysters on the ash reefs was highly variable, with smaller oysters (< 25 mm) dominating throughout and larger oysters rare (Fig. 5).

The 96 clam reefs displayed patterns similar to the Ash reefs in terms of over-all recruitment patterns and abundances (Fig. 6). Again, relatively low densities were found each sampling period. Recruitment events were followed by a sharp decline in oyster densities. Intertidal stratum had greater oyster densities than the other two tidal heights in all but two sampling periods (November

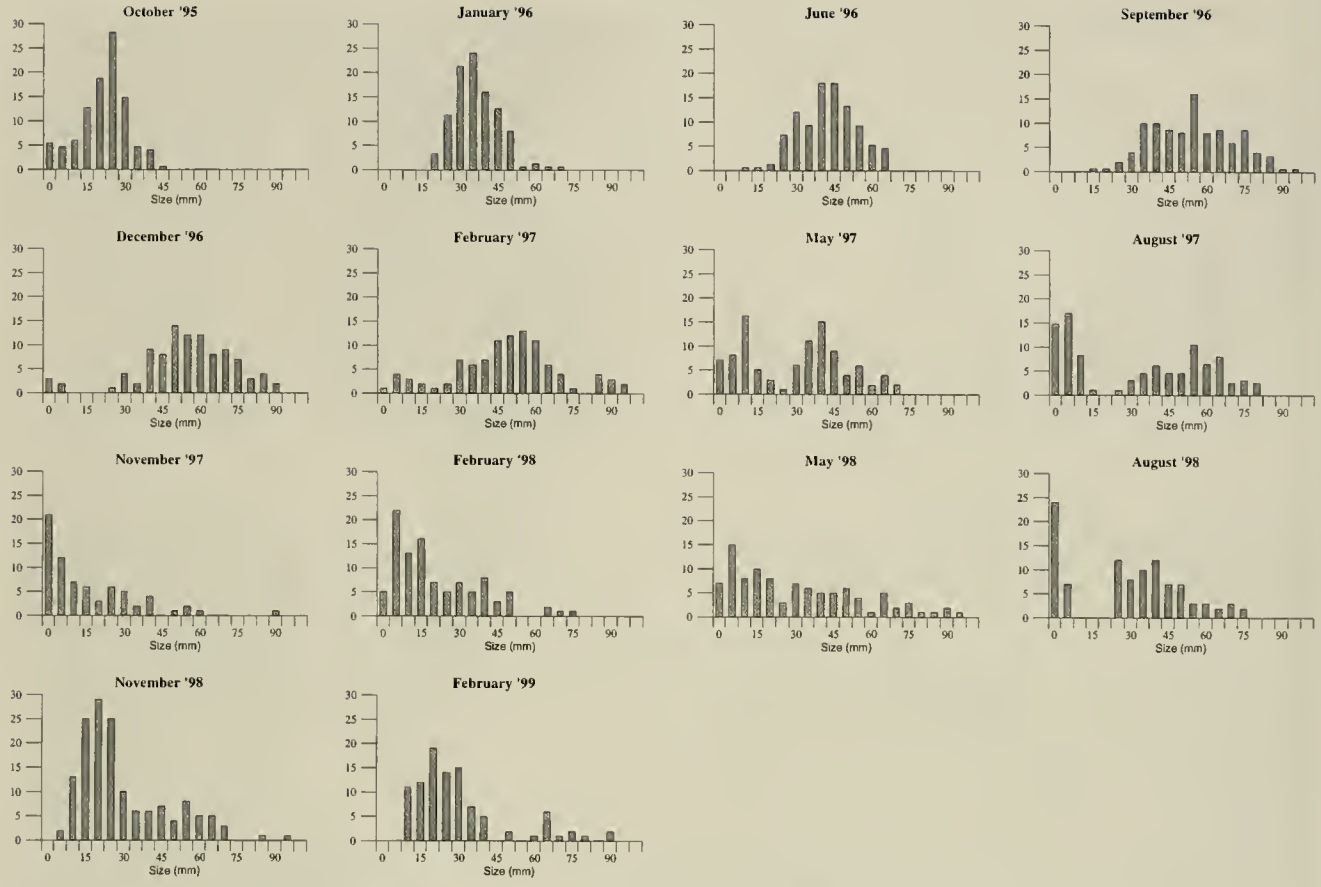

Figure. 3. Oyster size frequency distribution over the course of the study from the 95 Clam shell reefs. Size distributions were all animals combined from the three tidal heights.

1997 and August 1998). The size distributions within each sampling period was indicative of a population dominated by small oysters (< 20 mm; Fig. 7). However, in later sampling periods, there was a greater proportion (albeit small) of larger oysters on the 96 Clam reefs than found on the Ash reefs.

In 1996, in contrast to the low recruitment of oysters found on the reefs of coal ash and clam substrate, the Oyster reefs had a modest recruitment in December 1996 (Fig. 8). Survival on the oyster reef was greater than on the other substrate types, and again oyster densities were greater intertidally than at the other two tidal heights. The size distribution of oysters on the oyster shell reefs was approaching a unimodal normal distribution by May 1997 (Fig. 9). Recruitment events detected in November of subsequent years resulted in a bimodal size distribution. However, relatively large numbers of larger oysters persisted on the reefs.

Interstitial volumes differed significantly among the substrate types (Table 1). The oyster shell interstitial volume (0.7 L interstitial volume/1 L of substrate) was significantly greater than the volumes of both the clam (0.58 L) and coal ash (0.45 L) substrates. Analysis of variance of oyster densities from Subtidal samples detected significant differences among the Oyster, Ash, and Clam substrates (Table 2a). The Oyster substrate had significantly greater numbers of live oysters than the other reef types (Table 2a). The Intertidal samples from the 96 Clam reefs had significantly greater densities of oysters than the Subtidal samples (Table 2b). In addition, the densities of oysters found in the MLW samples were significantly greater than those found in the Subtidal samples on the Oyster shell reefs (Table 2c).

DISCUSSION

The reef bases at Fisherman's Island, Virginia, have all persisted, but quite different oyster populations have developed depending upon both the year of deployment and the substrate type used. Reduced elevations were observed in all reef bases, likely the result of some combination of subsidence, compaction, and erosion. Although interstitial volume estimates differed among the substrate types used on the 1996 reefs (Table 1), subsequent (mis) handling of the clam shells and large-scale production of the ash substrates (hence, poor quality control) resulted in additional compaction. These factors served to further the disparity between the oyster shell and the other substrates in terms of interstitial volume. This variation, we believe, had very significant consequences for the development of resident oyster populations as discussed below.

Oyster recruitment levels varied across the region over the duration of the study. As part of the ongoing yearly monitoring of oyster reproduction in the lower Chesapeake Bay, the Virginia Institute of Marine Science (VIMS) uses spatfall collectors (shellstrings) to determine patterns and levels of oyster recruitment (unpublished data, Virginia Oyster Spat Survey, 1970 to 1998, VIMS). During 1996 and 1997, recruitment estimated from the shellstrings at Fisherman's Island was lower in magnitude and later in each year compared with the 1995 shellstring results. This pattern was consistent with observations throughout the lower bay (Morales-Alamo and Mann 1996, Morales-Alamo and Mann 1997). Sampling on the reef surfaces was not timed specifically to record early postsettlement abundance. Other studies have shown

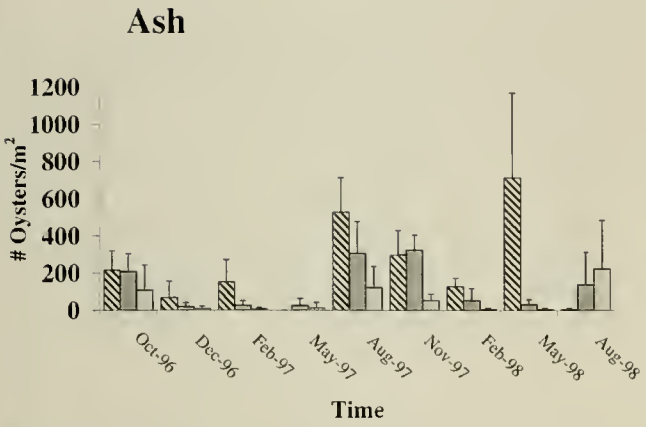

Figure. 4. Oyster abundance (number per m^2, mean ± SD) from three tidal heights throughout the study on the Ash pellet reefs planted in 1996. Legend as in Figure 2.

coal ash pellets (Alden et al. 1996, Andrews et al. 1997) and surfclam shells (Luckenbach unpublished data) are suitable substrates for oyster settlement. We would expect that early postsettlement densities, scaled to available substrate area, were comparable across reef type, but we lack confirming data.

Postsettlement survival of oysters varied in relation to tidal elevation, but the patterns were partially confounded by the loss of some tidal elevations from some reefs. The general trend observed was one of greater survival of oysters in the intertidal (Figs. 2, 4, 6, and 8), which is consistent with other studies conducted in the mid and southern Atlantic states of the U.S. (Kenny et al. 1990, Michener and Kenny 1991, O'Beirn et al. 1995, O'Beirn et al. 1996, Roegner and Mann 1995). Despite some variations in this pattern, significant differences were apparent for 96 Clam reefs, for which we have all tidal elevations present (Table 2b). In addition, oyster densities varied on the Oyster reefs between the two tidal heights evaluated (Table 2c). However, in the case of the Ash reefs, this trend was reversed on the final sampling period, with oysters virtually absent from intertidal samples (Fig. 4). These findings serve to highlight the importance of vertical relief when constructing oyster reefs in such environments as Fisherman's Island.

Variation in oyster abundance across substrate type was evident at all tidal heights (compare Figs. 4, 6, and 8), but because of missing levels on some reefs, statistical comparisons by substrate type were made only for the subtidal level (Table 2a). The significant trend of greater abundance of oysters on the Oyster reefs compared to the Ash reefs and 96 Clam reefs at this tidal level was evident throughout. Over-all mean density on the Oyster shell reef ($935/m^2$) exceeded that on the 96 Clam shell reef ($149/m^2$) and the Ash reef ($141/m^2$) roughly sixfold. Visual comparisons of the reefs are even striking. The Oyster shell reefs supported an uninterrupted layer of live oysters, which was not apparent on the other substrates, both of which had only sporadic clusters of oysters. In addition, the clam shell and coal-ash pellets reefs mostly retained

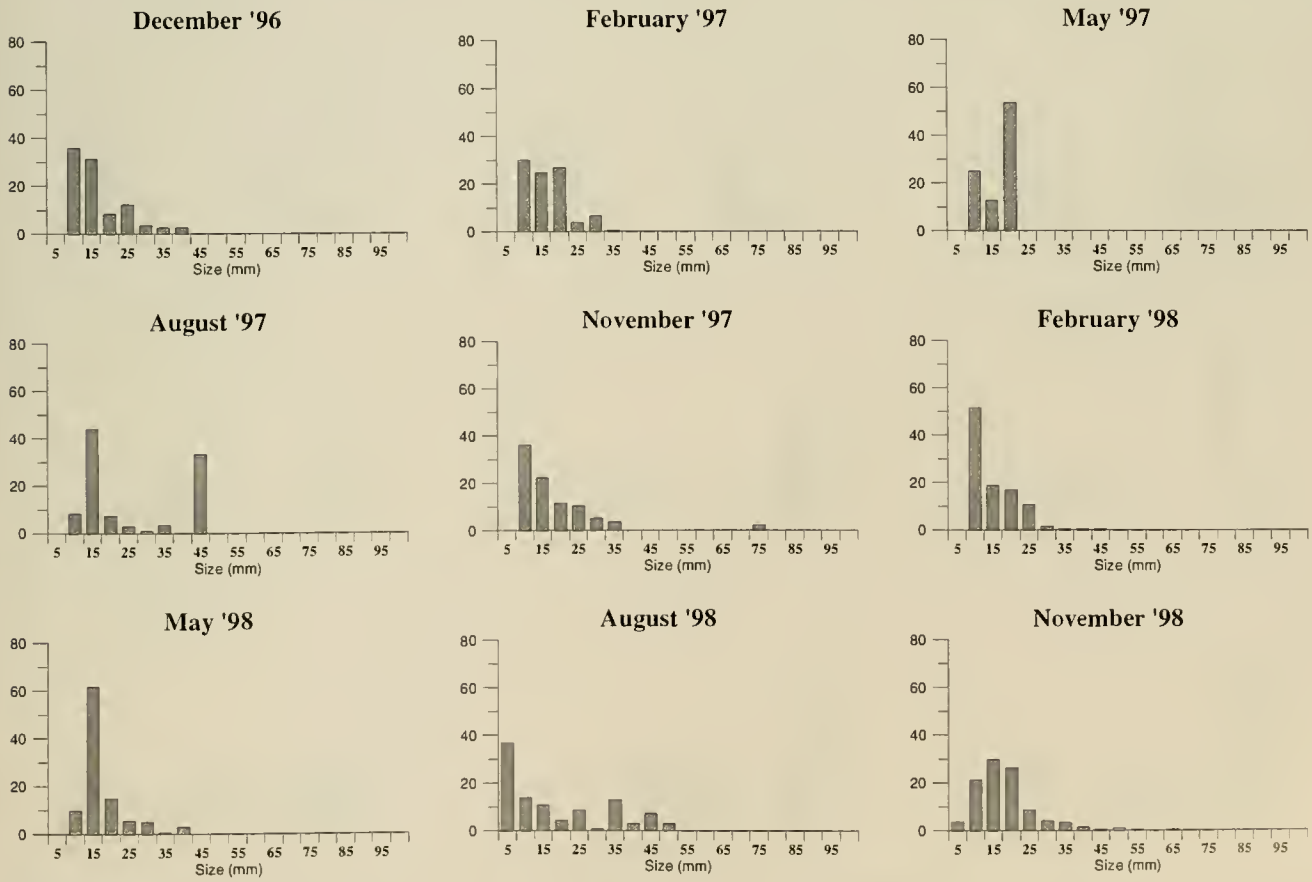

Figure. 5. Oyster size frequency distribution over the course of the study from the Ash reefs planted in 1995. Size distributions were all animals combined from the three tidal heights.

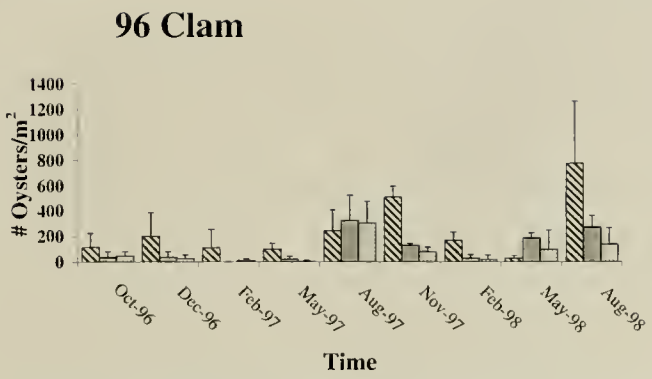

Figure. 6. Oyster abundance (number per m^2, mean ± SD) from three tidal heights throughout the study on the 96 Clam shell reefs. Legend as in Figure 2.

their original bleached white and dark gray colors, respectively, throughout the study, which is indicative of little or no biotic development on the reefs.

The dominance of the oyster shell substrate was further underscored when examining the size data of oysters from each of the substrate types. Small oysters (< 20 mm) dominated both the Ash and 96 Clam substrates (Figs. 5 and 7) throughout the entire monitoring period. There was no persistence of larger (older) oysters in either of these reef types. The 95 Clam reefs and the Oyster shell reefs had relatively greater proportions of larger oysters representing multiple year classes (Figs. 3 and 9). In August 1998, 22% (138 oysters/m^2) of the standing stock of oysters on the Oyster shell reefs had shell height ≥ 60 mm. This represented a substantial number of larger oysters that could contribute considerably to future reproductive events (Cox and Mann 1992) and, therefore, realizes a primary goal of the restoration efforts. In addition, the higher density of oysters resulted in a reef matrix that is likely to ensure the maintenance and stability of the valuable interstices.

We suggest that several factors related to the availability of interstitial space account for the observed differences in oyster abundance across the reefs. First, the reduced interstitial volume in the ash pellets and clam shell relative to oyster shell may have reduced the amount of surface area available for settlement. Bartol and Mann (1999) have reported oyster settlement onto shells 10–15 cm below the surface in a constructed reef in the Piankatank River, Virginia, and J. Nestlerode and F. O'Beirn (unpublished data) have made similar observations in substrate baskets buried in these reefs at Fisherman's Island. The density estimates we report here include oysters collected to a depth of 15 cm scaled to a flat surface area of the reef and do not account for subsurface area that might be available for oyster attachment. Thus, oyster settlement onto the Oyster shell reefs may have exceeded those on the Ash and 1996 Clam shell reefs. Because recruitment levels were low, however, and attachment surface was not in limited supply, it is unlikely that settlement differences accounted for most of the variation across reef type.

Differential mortality of oysters at the surface and below the

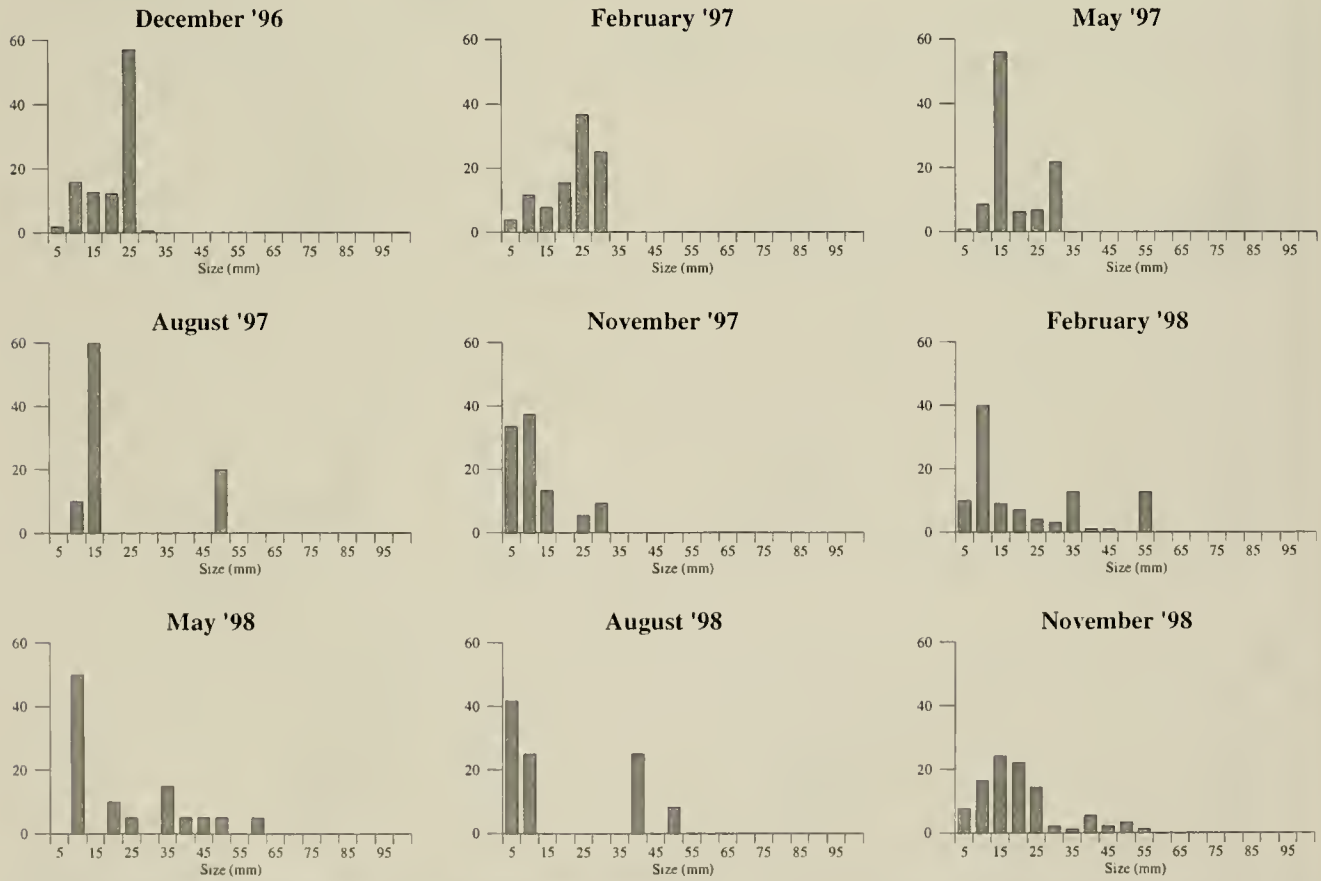

Figure. 7. Oyster size frequency distribution over the course of the study from the 96 Clam shell reefs. Size distributions were all animals combined from the three tidal heights.

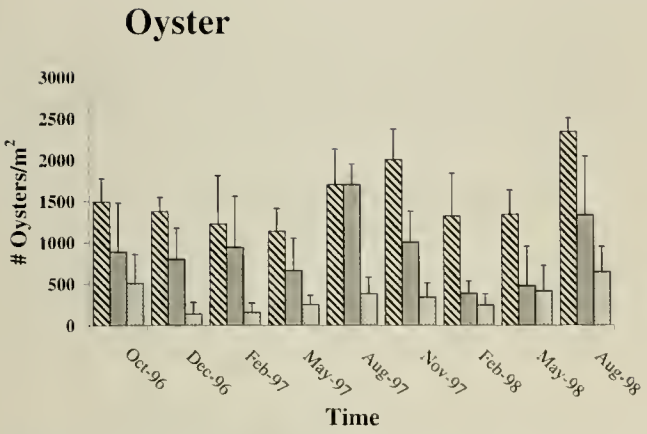

Figure. 8. Oyster abundance (number per m^2, mean ± SD) from three tidal heights throughout the study on the Oyster shell reefs planted in 1996. Legend as in Figure 2.

surface of the reefs is a likely explanation for the abundance patterns we observed. Bartol and Mann (1999) have demonstrated the value of interstitial space in aiding the survival of young oysters. The refuge afforded by the interstices protects the young oysters from predation and buffers them from climatic extremes. The considerably lower levels of interstitial space located on the clam shell and ash reefs most likely resulted in increased exposure of the young oysters to potential predators and other detrimental environmental factors (see reviews by Shumway 1996, White and Wilson 1996).

Finally, we expect a degree of positive density dependence in the development of oyster populations on constructed reefs. If the initial settlement and survival of oysters is sufficient (in part because of factors above), living oysters come to dominate the surface features of the reef and contribute to further interstitial space. In effect, the oysters themselves provide a refuge in numbers. In addition, the presence of large numbers of resident oysters in subsequent years may enhance settlement through the release of water-soluble settlement-inducing peptides (Tamburri et al. 1992, Turner et al. 1994). For example, the large recruitment event in 1995 (Fig. 2) was sufficient to result in a veneer of living oysters covering most of the clam shell substrate. Thus, when a smaller recruitment event occurred in 1996, the 95 Clam reefs and the 96 Clam reefs presented quite different habitats for new recruits and both recruitment and survival were greater on the older clam shell reefs (compare Figs. 2 & 3 with Figs. 6 & 7). Similarly, the abundances of oysters and spatial complexity of the oyster shell reefs have been increasing since their planting in 1996. Both the 96 Oyster shell reefs and the 95 Clam shell reefs developed abundant oyster densities, with multiple year classes present and reef surfaces dominated by living oysters. In contrast, the Ash reefs and the 96 Clam reefs have failed to develop abundant oyster populations, and generally only supported small size classes, which diminished in abundance after recruitment events.

Our findings suggest that in areas and years with high oyster

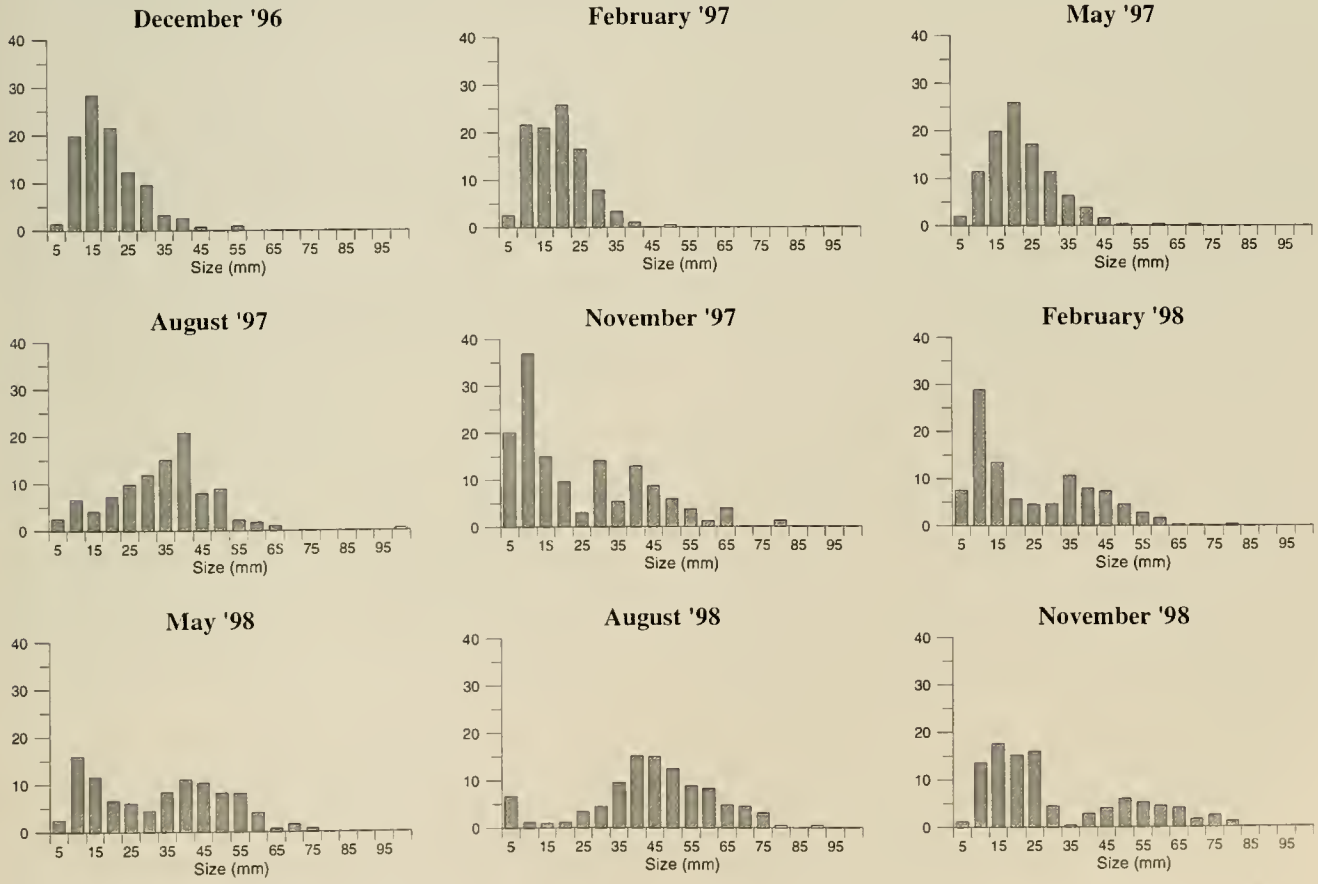

Figure. 9. Oyster size frequency distribution over the course of the study from the Oyster shell reefs planted in 1996. Size distributions were all animals combined from the three tidal heights.

TABLE 1.

ANOVA and Tukey HSD tests on interstitial space obtained from the three substrate types.

ANOVA: Interstitial Volume by Substrate Type				
Source	df	SS	F Value	*P*-value
Substrate	2	0.156	42.8	0.0001
Error	12	0.178		
Tukey test		Oyster	Clam	Ash
Mean volumes (SD)		0.7 L (0.04)	0.58 L (0.06)	0.45 L (0.02)

Interstitial volume given as interstitial volume in liters per 1-L substrate.

TABLE 2.

Results of the ANOVAs and Tukey HSD tests on (a) oyster abundance according to substrate type, (b) oyster abundance at tidal heights on clam reef, and (c) oyster abundance at tidal heights on oyster reefs.

(a) ANOVA: Oyster Abundance by Substrate Type (Subtidal Elevations Only)

Source	df	SS	F Value	*P*-Value
Substrate	2	74.39	28.09	.0001
error	43	56.94		
Tukey test:	Oyster		Clam Ash	

(b) ANOVA: Oyster Abundance by Tidal Height (Clam Shell Reefs Only)

Source	df	SS	F Value	*P*-Value
Tidal height	2	14.85	4.01	.0263
error	38	70.35		
Tukey test:	Intertidal		Mean Low Water	Subtidal

(c) ANOVA: Oyster Abundance by Tidal Height (Oyster Reefs Only)

Source	df	SS	F Value	*P*-Value
Tidal height	1	8.99	29.86	.0001
error	26	7.83		
Tukey test	Intertidal		Mean Low Water	

Tukey Test given in descending order of magnitude.

recruitment rates, the nontraditional substrates used here can serve as suitable base materials for restoring oyster reefs if mounded to provide sufficient vertical relief. In low recruitment environments, however, it is important that adequate interstitial space be present to support oyster survival. In the present study, only oyster shells provided adequate interstitial space for the development of an oyster population in low recruitment years. Given our initial concern that oyster shells are in short supply throughout much of the mid-Atlantic region of the U.S. and the unpredictable nature of recruitment in many areas, we are led to ask how to best use available substrates for reef restoration. Repeated handling of surfclam shells—from the shucking house to reef construction—seems assured of resulting in fragmentation and the tight packing on reefs described above. Mixed shell plantings using surfclam shells in combination with other shell (e.g., whelks and hard clams) may support better development of oysters by reducing compaction and increasing available interstitial space (J. Wesson, Virginia Marine Resources Commission, pers. comm). Improved quality control in the production process of coal ash pellets could result in more uniform-sized pellets, similar to those used by Andrews et al. (1997), which had a mean diameter ≈ 5 cm, provided greater interstitial space, and supported good oyster survival. Perhaps the greatest impediment to the use of coal-ash pellets in future oyster reef restoration efforts results from the U.S. Federal Highway Act of 1995, which mandated the use of recycled material in roadbed construction; thereby, changing coal ash from a waste product into a commodity and increasing its cost.

A variety of alternative substrates for oyster settlement have been tested in other studies including slate (Haven et al. 1987), expanded shale, shredded tires (Mann et al. 1990), gypsum, *Rangia cuneata* shells, limestone, concrete, and gravel (Soniat et al. 1991, Haywood and Soniat 1992, Haywood et al. 1999). Varying degrees of suitability were observed for the different substrate types. In North Carolina, limestone marl is a routinely used settlement substrate in a fishery enhancement program (Marshall et al. 1999). The applicability of these substrates for large-scale endeavors may have to be re-evaluated in light of the findings presented in this study, particularly as they relate to substrate stability and interstitial volume.

The construction of reef structures in order to promote shellfish restoration represents a significant investment of public and private resources. Developing protocols that help maximize ecological return on this investment will be important for future efforts to restore oyster reef, as will evaluating these design and construction protocols on sufficiently large spatial and temporal scales. We have observed an interaction between the substrate used in the construction and oyster recruitment levels in the development of oyster populations on large-scale constructed reefs. During periods of low natural recruitment, only substrates that provide adequate interstitial space (oyster shell in the current study) are sufficient to support the development of a viable reef. During periods of high recruitment, poorer quality substrate (i.e., that providing less interstitial space) may prove sufficient as the newly recruited oysters themselves serve as ecosystem engineers (Jones et al. 1994) providing physical refuge. In temperate, polyhaline environments, the provision of vertical relief is important in ensuring oyster survival. Again, the combination of substrate placement and oyster recruitment, survival, and growth interact to affect restoration success. Therefore, restoration design criteria (e.g., the actual configuration of interstitial space and degree of vertical relief) must account for both geophysical (e.g., siltation and ice scour) and biological (e.g., subtidal and intertidal predators) mechanisms. Given these potential constraints, we appreciate that the many factors influencing oyster survival and growth, and hence a successful start to restoration efforts, have yet to be elucidated.

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THE GULF COAST OYSTER INDUSTRY PROGRAM: AN INITIATIVE TO ADDRESS INDUSTRY'S RESEARCH NEEDS

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ABSTRACT The Gulf Oyster Industry Program (GOIP) was created in response to petitions from the Louisiana Oyster Task Force and Gulf Oyster Industry Council. These organizations initially sought long-term, research-based assistance through the Louisiana Sea Grant College Program. Subsequently, they worked with the Gulf region's Sea Grant network, the National Fisheries Institute, and the congressional delegations of several Gulf states to have the GOIP established as part of the National Sea Grant College Program in the National Oceanic and Atmospheric Administration. Implementation of this new program in a competitive funding environment necessitated reconciling two, sometimes conflicting, management goals: (1) projects having the greatest utilitarian benefits as judged by the oyster industry stakeholders; and (2) projects having the greatest scientific merit as determined by expert peer reviewers. Program development, implementation, and outcome of the first-year proposal solicitation and selection process are presented.

KEY WORDS: Gulf, oyster, research, programming

INTRODUCTION

The Gulf region leads all other regions of the U.S. in oyster production (NMFS 1998); however, the nation's total annual production of molluscan shellfish has been declining steadily. As in other areas, oyster producers in the Gulf states face myriad problems associated with (1) the presence of opportunistic bacteria, especially vibrios, in oyster-growing waters; (2) multiple-use conflicts in traditional oyster grounds, especially those associated with coastal restoration projects; (3) pollution from upstream urban and industrial development, recreational camps, and oil production facilities; (4) the depletion of harvestable stocks by oyster predators and diseases, especially the parasite *Perkinsus marinus*; (5) uncertainty about long-term stability of oyster leasing policies; and (6) declining profitability caused by declines in seasonal meat yields, a changing work force, stringent new regulations, and limited technological options.

In response to a request from the legislatively appointed Louisiana Oyster Task Force (LOTF) and the Gulf Oyster Industry Council (GOIC), the Louisiana Sea Grant College Program developed and implemented the Gulf Oyster Industry Initiative, a long-term, research-based plan to assist the industry achieve full economic recovery. In 1997, the industry successfully petitioned Congress to support the initiative with $1 million per year for 5 years. Administered by the National Sea Grant College Program, the money is to fund competitive grants through Sea Grant universities around the country. The resulting program combines the scientific knowledge of highly qualified academic researchers with the experience of industry and management agency personnel in a coordinated, comprehensive search for viable solutions to the most pressing problems of the Gulf's oyster industry. Although the Sea Grant programs of the Gulf region have supported many oyster-related projects during the last 25 years, this particular undertaking is novel in its scope, management, and the close working relationships between scientists and industry stakeholders.

The program's objectives are (1) to assist the oyster industry with needs analyses and prioritization; (2) to seek credible scientific viewpoints and responses to those needs; (3) to educate the Gulf oyster industry about the scientific status of their issues; and (4) to facilitate the development and funding of research proposals to address those issues.

APPROACH

The GOIP was designed to have continuous involvement of the oyster industry. In the program's first year, the research priorities were established by an Industry Advisory Panel (IAP) and described in the Request for Preliminary Proposals (RFP). The panel consisted of an oyster harvester and a processor from each Gulf state, ensuring that both viewpoints would be recognized. The panelists were selected by the directors of Gulf coast Sea Grant programs, with recommendations from the GOIC and the LOTF.

After preliminary proposals were received from academic researchers, the IAP determined which ones should be selected for solicitation as full proposals. The full proposals were subsequently evaluated by a Scientific Review Panel, which comprised representatives from the oyster industry, the National Sea Grant Office, and a Gulf state shellfish management agency, and scientists familiar with contemporary oyster research. This panel selected proposals for possible funding on the basis of their scientific merit and cost effectiveness, and provided a general ranking of the proposals based on peer reviews and the panelists' individual knowledge of the subject matter.

RESULTS

Primary consideration for funding was given to proposals addressing research topics previously identified as critical to the continued viability of the oyster industry. In the GOIP's inaugural year (FY 98), 18 full proposals totaling $1.1 million were solicited after a review of 35 preliminary proposals totaling $3.9 million in requested funds. Ten proposals were ultimately funded, totaling approximately $868,000. The grant recipients were from Virginia, North Carolina, Florida, Alabama, Mississippi, Louisiana, Texas, and California. The research topics and examples chosen by the IAP follow; projects receiving funding in that topic are described.

Pathogenic Organisms

A perception that the consumption of raw oysters is hazardous to public health is reducing the demand for shellstock oysters and

inflicting severe economic distress on oyster growers, harvesters, and processors. Suggested research topics include:

- develop means of treating shellstock and/or raw oyster meats to eliminate human pathogens;
- develop and evaluate strains of transgenic oysters that are capable of destroying pathogenic organisms;
- develop an international database on *Vibrio parahaemolyticus* to centralize information on the organism;
- develop depuration methods for removing vibrios from oysters; and
- develop quick depuration methods using probiotics

1998 Projects

Project Title: Use of GRAS compound, Diacetyl, for the removal of *Vibrio vulnificus* from shellstock and shucked oysters.

The objectives of this project are to determine the effectiveness of diacetyl for the reduction and/or elimination of naturally occurring populations of *V. vulnificus* within shellstock and shucked oysters and to investigate the conversion of opaque morphotypes of *V. vulnificus* to the translucent morphotype in both oyster products.

Funding level: $39,564 GOIP and $20,480 match for Year 1. $38,763 GOIP and $21,424 match for Year 2.

Consumer Attitudes and Preferences

Consumption of oysters has fallen significantly during the past decade. Lack of knowledge concerning the attitudes, preferences, and other characteristics of potential oyster consumers is perceived as an obstacle to recovering traditional markets and expanding demand for new and traditional oyster products. Suggested research topics include:

- determine oyster consumer demographics, consumption patterns, attitudes, and preferences;
- develop news media protocols for researchers and state regulatory personnel;
- develop and market-test new oyster products; and
- determine the market characteristics, including sale (i.e., region, size of establishment, average sales, etc.), distribution, and preferred product forms.

Oyster Disease

Incidences of oyster disease are rising dramatically in Gulf oyster grounds, and much of the region's oyster industry is at risk of severe economic loss through mortality of harvestable oysters. Possible research topics include:

- develop and evaluate genetic strains of oysters with superior growth and disease (pathogen) resistance; and
- develop technology to produce quantities of oyster larvae with superior growth and disease resistance adequate for commercial operations.

1998 Projects

Project Title: Predicting time to critical levels of the oyster disease *Perkinsus marinus*: A new tool for increasing oyster production.

The objectives of this project are to determine monthly levels of *P. marinus* in Galveston Bay oysters, predict the time needed to reach a critical level of disease, evaluate the reliability of the predictions, and use the information to increase oyster production by moving or harvesting oysters before they are killed by disease.

Funding level: $55,229 GOIP and $27,365 match for Year 1. $54,229 GOIP and $27,365 match for Year 2.

Project Title: Optimization of gene delivery for improved oyster health.

The objectives of this project are to compare commercial lipofection reagents to determine which is most effective; evaluate lipofection of sperm, eggs, and embryos; and evaluate the expression and function of transferred genes by disease challenging larval and adult oysters with *Perkinsus marinus*.

Funding level: $60,307 GOIP and $30,153 match for Year 1.

Project Title: Creation of an oyster cell line for *Crassostrea virginica* (Gmelin).

The objectives of this project are to produce retroviral vectors that express different reporter genes and proto-oncogenes from appropriate promoter elements in oyster cells; to optimize conditions for the infection of primary cultured cells from *C. virginica*, to infect primary cultures established from both *C. virginica* hearts and from enzymatically disrupted early embryos with the transforming retroviral vectors, to characterize the growth characteristics and phenotype of immortalized cell lines, and to test cultivation of the oyster pathogens *Haplosporidium nelsoni* and *Perkinsus marinus* in the oyster cell lines.

Funding level: $76,098 GOIP and $37,195 match for Year 1. $88,889 GOIP and $56,930 match for Year 2.

Coastal Restoration, Freshwater Diversion

Coastal land loss, deterioration of estuarine habitat, and coastal restoration programs (e.g., freshwater diversions, and sedimentation projects) are causing widespread dislocations and conflicts with established oyster-producing operations. Suggested research topics include:

- educate oyster farmers, public officials, and citizens regarding the economic role of the oyster industry and the economic costs of displacing and relocating oyster bedding operations;
- develop and test freshwater diversion and oyster farming strategies that take account of temperature and salinity conditions likely to promote fouling of bedded oysters by the hooked mussel (*Ischadium recurvum* [Rafinesque]).

1998 Projects

Project Title: The Caernarvon Freshwater Diversion Project and oyster farmers: what happened and what it means.

The objectives of this project are to describe the Louisiana oyster relocation program after recent court cases and political activity, allowing oyster farmers to make pro-active decisions about opting into the lease relocation program as it currently exists.

Funding level: $30,666 GOIP and $15,989 match: 1 year project.

Labor and Mechanization

The traditional labor base that supports oyster growing, harvesting, and processing is shrinking rapidly, with consequently declining production and increased costs. Suggested research topics include:

- investigate and develop cost-effective mechanized approaches to oyster harvesting, processing, and packing; for example, oyster shucking and harvesting machinery.

1998 Projects

Project Title: Technical and economic evaluation of a freeze-heat-cool process that facilitates oyster shucking.

The objectives of this project are to develop and evaluate pro-

totype equipment to test the freeze-heat-cool process that will facilitate shucking of Gulf oysters.

Funding Level: $61,390 GOIP and $31,000 match for 1 year.

Genetics, Hatchery Production

Seasonal monetary losses from the processing of oysters with poor meat yield hinder economic stability and growth of the industry. Some states have areas suitable for growing oysters, but these are not used because of traditional or regulatory restraints against moving seed oysters from public to private beds. These areas have high potential for oyster culture using hatchery-produced oyster seed. Suggested research topics include:

- develop cost-effective hatchery, nursery technology to augment wild oyster production by producing specialized strains; and
- address practical problems, such as fouling, predation, disease, that may be common to oyster production in general but are more acute in a farming situation.

1998 Projects

Project Title: Natural dermo resistance and its role in the development of hatcheries for the Gulf of Mexico.

The objectives of this project are to use putatively resistant broodstock from both the Gulf of Mexico and Chesapeake Bay and determine the inherent resistance of their progeny to Dermo disease to provide clear evidence for the existence of 'naturally resistant populations' of American oysters and to justify the continued development of these stocks by industry.

Funding level: $41,591 GOIP and $20,972 match for Year 1. $42,693 GOIP and $21,772 match for Year 2.

Project Title: Optimum size for planting hatchery-produced oyster seed.

The objectives of this project are to determine the optimum size of hatchery-produced oyster seed for planting on bay bottoms and to relate optimum size to costs for producing various size seed.

Funding level: $31,737 GOIP and $17,249 match for Year 1. $31,993 GOIP and $17,766 match for Year 2.

Hooked Mussel Fouling

Hooked mussel fouling on oyster growing areas has drastically increased harvesting costs by requiring the laborious removal of mussels from marketable oysters or further transplanting oysters to areas with higher salinity. Suggested research topics include:

- determine predator–prey relationships;
- determine the effects of varying haline and aerial exposures to mussel attachment; and
- determine mussel life cycle and/or recruitment.

Harmful Algal Blooms

The first reported occurrence of red tide in the central Gulf region caused a lengthy public health closure, halting production for weeks and causing severe economic hardship in the affected area. A possible research activity is to develop rapid detection methods for red tide.

1998 Projects

Project Title: Ozone-assisted depuration of red-tide-contaminated shellfish and seawater.

The objectives of this project are to determine the optimal amount of ozone needed to inactivate the brevetoxins in contaminated clams and oysters in recirculating seawater depuration tanks; determine the length of time required at the optimal amount of ozone for the desired reduction of toxins to safe levels in shellfish; and determine the effect on shelf-life of red-tide-contaminated clams and oysters subjected to such depuration.

Funding level: $52,575 GOIP and $20,307 match for Year 1. $52,575 GOIP and $20,307 match for Year 2.

Point-Source Pollution

Known point sources of pollution negatively affect certain potential oyster-growing waters, with consequent public health risks and loss of revenue to growers. Public health closures of oyster grounds in restricted areas are costly to the Gulf states in terms of lost resources, employment, and revenues. Possible research topics include:

- evaluate alternative uses of recalled oysters;
- develop a process for identifying pollution sources and linking clean-up to closure of beds.

1998 Projects

Project Title: Determination of design and operational criteria for a marshland upwelling system to treat domestic wastewater from coastal camps.

The objectives of this project are to characterize influent wastewater parameters, to determine effectiveness of a marshland upwelling system to remove fecal coliform and *E. coli* from raw wastewater, to determine the extent and movement of the freshwater plume that develops over time, and to develop an educational program for marine advisory agents and potential users.

Funding level: $78,548 GOIP and $25,569 match for 1 Year.

Project Title: Legal authority to clean up oyster beds closed because of pollution.

The objectives of this project are to examine both the applicability and inapplicability of state and federal Clean Water Act programs to compel clean up of shellfish beds contaminated by point-source pollution.

Funding Level: $30,666 GOIP and $15,989 match: 1 year project.

Black Drum Predation

Restrictions on use of gill nets as harvesting gear for finfish have enabled black drum populations to flourish, with a consequent increase in oyster predation by that species. A possible research activity is to develop novel methods of deterring black drum predation.

New Priority for FY99–00

The IAP added an additional topic for the FY99–00 preliminary proposal solicitation: economic impacts of regulatory action. The regulation of molluscan shellfish is unique compared with all other foods. Regulatory action either by state or federal public health agencies and subsequent news media responses can have severe economic impacts on the harvesting, processing, and marketing of shellfish, such as Gulf oysters. Suggested research topics include:

- analyses of de-listing of a processor or state from the Interstate Certified Shellfish Shippers List;
- analyses of inaccurate media reports on sales; and
- analyses of ramifications from product disparagement.

DISCUSSION

The scope of the projects awarded for 1998 is somewhat surprising considering the oyster industry's involvement in the program, because some of the research is basic in nature. Of the approximately $868,000 awarded for FY98–99 projects, 39% supported oyster disease research, 17% addressed oyster hatchery and genetics research, 24% investigated point-source pollution and harmful algal blooms, 7% was for labor and mechanization needs, and 3% focused on coastal restoration topics. Most surprising is that only 9% of the total awards addressed pathogenic organisms, such as *Vibrio vulnificus*, an issue perceived widely as having the greatest negative impact on the Gulf oyster industry. Despite the desperate problems Gulf oyster farmers are having with hooked mussels and black drum predation, no proposals were awarded in those categories during the program's first fiscal year. These results reflect how the GOIP solicitation and selection processes focused researchers on the industry's needs, and selecting the most highly ranked proposals based on their scientific merit.

It is apparent that the GOIP is addressing several important topics identified in earlier studies of oyster industry problems. Several of the research topics identified in the Gulf states' regional oyster management plan (GSMFC 1991) and a plan addressing the restoration of the American oyster industry (Anonymous 1990), which engendered the National Oyster Disease Program administered by the National Sea Grant Office, are being addressed by GOIP projects. The GOIP is distinguished by the way in which it addresses industry's annual needs assessment and prioritization. The greatest programmatic challenge is to fund proposals that not only address industry's needs but also have high scientific merit and educate the industry.

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A REVIEW OF SHELLFISH RESTORATION AND MANAGEMENT PROJECTS IN RHODE ISLAND

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ABSTRACT Shellfish management and restoration efforts in Rhode Island date back to the late 19th century. From the late 1890s to the Second World War the Rhode Island Fisheries Commission operated a lobster hatchery in Wickford Harbor in response to a perceived decline in lobster catches in Narragansett Bay. Berried lobsters were collected, eggs hatched, larvae reared, and postlarval fifth stage juveniles were released to the bay. The project was discontinued primarily because of costs and a failure to demonstrate the efficacy of juvenile seeding in improving lobster catches. From the 1930s to the 1980s, there have been several similar efforts to establish hatcheries to produce juvenile bivalve mollusks for public and private reseeding efforts, but none of these efforts were economically sustainable. The longest running efforts to improve shellfisheries have been state programs to relay northern quahogs, *Mercenaria mercenaria*, from dense population assemblages in waters closed to shellfishing. Large-scale relays began in the 1950s in response to heavy fishing pressure but ended in the 1960s when commercial power dredging for shellfish was banned in Narragansett Bay. A small-scale state program existing since the late 1970s pays a modest fee to supervised shellfishers for hand digging quahogs in closed waters and planting them in management areas for depuration and eventual harvest. The amounts of shellfish relayed annually has varied widely since 1977, ranging between 7 and 322 metric tonnes, with an average of 98 metric tonnes per year. A new relay program has been underway since 1997. It involves assessing the shellfish stocks in the closed Providence River and hiring dredge boats to relay shellfish into down bay management areas. Based on maximum sustainable yield (MSY) considerations, annual relays should not exceed 10.3% of the standing crop (or 2721 metric tonnes) in the Providence River. An effort to restore lobsters onto monitored artificial reefs is underway using settlement funds from a 1989 oil spill in Narragansett Bay. Finally, the Rhode Island Public Benefit Aquaculture Project, a joint educational effort with commercial fisheries involvement, is involving secondary level students in the nursery culture of shellfish (though marina-based upwellers) for seeding of public shellfish beds.

KEY WORDS: Shellfish restoration, Rhode Island, shellfish relay, shellfisheries, Narragansett Bay

INTRODUCTION

Since the King Charles Charter of 1663 uniting the Rhode Island Colony of Newport to the mainland colony of Providence Plantations, there has been a codified recognition of the importance of fish and fisheries to all the citizens of Rhode Island. Although the charter is best known for its early establishment of religious freedoms, it also set forth the first principles of a public trust doctrine by entrusting the stewardship of coastal waters to the colonial assembly. All citizens were assured of free access to the waters for fishing and the collection of seaweed. In 1842 these public trust principles were incorporated directly into Article 1, Section 17 of the state constitution, and they now form the basis of all fisheries and coastal management efforts undertaken in Rhode Island's tidal waters. Nixon (1993) provides an overview and analysis of Rhode Island's public trust doctrine as it relates to shellfisheries and aquaculture in coastal waters.

The history of Rhode Island's shellfisheries can be broadly characterized as having three distinct periods. During the first period, which began in pre-Colonial times with the Narragansett Indians, shellfishing was usually a summer activity. Roger Williams (1643) the founder of Providence Plantations Colony noted that the Narragansetts would ". . . wade deepe (sic) and dive . . ." for oysters and quahogs. Shell middens found along the shore of Narragansett Bay, notably in the Potowomut area of what is now Warwick, are testament to the importance of shellfishing in the pre-Colonial era. Even the scientific name of the northern quahog—*Mercenaria mercenaria* (Linnaeus, 1754)—is testimony to the fact that the white and purple beads made from their shells were an important trading currency. From the Colonial period and early statehood until the Civil War, shellfisheries were essentially subsistence or small-scale commercial operations as authorized under the King Charles Charter or under the Article 1, Section 17 provisions of the 1842 Rhode Island State Constitution. Oysters were harvested as feed for swine and for storage as a personal food item during winter. The shells were burnt to produce lime (Kochiss 1974).

The second major period in Rhode Island's shellfisheries began with the passage of the Oyster Act of 1864 and the establishment of the Rhode Island Shellfisheries Commission. This act of the legislature allowed, for the first time, the leasing of tracts of submerged public trust lands for the purpose of cultivating oysters (Nixon 1993). The early oystermen in Rhode Island readily recognized that the waters in Narragansett Bay were very good for

growout, or maturation, of oysters (*Crassostrea virginica*). But, the seed beds in the state were not particularly productive and recruitment was very sporadic, so tons of seed oysters were brought annually into Narragansett Bay from Long Island Sound and as far away as the Chesapeake Bay (Hale 1980). This massive transplantation of oysters onto extensively managed aquaculture beds might be considered Rhode Island's first successful program of shellfish restoration, albeit the direct beneficiaries were the oyster leaseholders. In 1910, during the height of Rhode Island's oyster aquaculture industry, 8100 ha of Narragansett Bay was leased to private companies for oyster culture and 7000 metric tonnes of oysters were harvested annually (NMFS landing statistics as cited in Olsen and Stevenson 1975).

Throughout the period of massive oyster aquaculture leases in Narragansett Bay, state efforts to boost shellfish production included programs to monitor and control shellfish predators. The former Rhode Island Shellfisheries Commission and the oyster companies initiated an annual starfish census. These are reliable estimates of starfish populations in Narragansett Bay from 1880 to 1940 (Pratt et al. 1992). From time to time—when the predator populations became particularly high—there were starfish "bounty" programs (Hale 1980), and as part of regular oyster bed maintenance the oyster vessels were rigged with starfish mops similar to those still used on oyster beds in Connecticut (Olsen et al. 1980). The old-time oystermen recognized that predator control programs were a very effective way to increase shellfish production, but there was a lack of understanding that starfish could regenerate from body parts. One common practice of starfish "control" was to cut landed starfish in half and throw them overboard (Luther Blount, former President of Warren Oyster Company, pers. comm. 1993).

Beginning in the 1920s, the oyster aquaculture industry began a slow decline, culminating in a near collapse following the Great Hurricane of 1938. A number of causes have been attributed to the decline of the oyster industry in Rhode Island. These include changes in upland land uses and increased sedimentation of prime beds (Hale 1980), increased metal pollution due to a burgeoning metal-plating industry (Nixon 1995), and increased eutrophication and hypoxia in the upper reaches of Narragansett Bay due to sewage disposal (Desbonnet and Lee 1991). The Great Hurricane of 1938 was certainly a major blow to the oyster industry. Most of the shoreside docking and processing facilities were severely damaged by the storm (Olsen et al. 1980), and the recovery of the industry was hampered by the lack of a readily available workforce due to the onset of World War II (Hale 1980).

Another reason for the decline and eventual demise of Rhode Island's oyster industry may lie in the major socio-political changes that occurred in Rhode Island during the mid-1930s. In many ways, Rhode Island's oyster industry was a product of the "mill town" social system that grew up during the Industrial Revolution and the heyday of Rhode Island's textile industry in the late 19th and early 20th centuries. The oyster industry, as it was constituted, was extremely labor-intensive, very much like other industries of the era. Beginning in 1935, political shifts in the state government (known locally as Gov. T. F. Green's bloodless revolution) toward more populist policies may have had some impact upon the oyster industry. McLoughlin (1978) argued that this change in political philosophy had a profound impact on the textile industry and other industries that failed to adapt in a changing political climate.

The third period of Rhode Island's shellfisheries, reviewed by Hale (1980) and Boyd (1991), arose immediately after World War II. Many of the troops returning from Europe or the Far East in the 1940s began tonging for quahogs, largely because the old oyster beds were no longer tended and the oyster companies were not hiring. In the late 1940s the keyport bullrake was invented in the Mid-Atlantic region and was quickly introduced to Rhode Island. After several technical refinements, the bullrake became the most widely used commercial shellfishing implement by the 1960s (Boyd 1991).

The two key controversies in the shellfisheries during the 1940s and 1950s were fees paid by fishermen to oyster leaseholders for the privilege of fishing on the beds, and the introduction of power dredges for harvesting quahogs. When oyster production on the old leases declined, there was little or no effort by the state to revoke the leases and return the grounds to the open fisheries. Only after the demise of the last Rhode Island oyster company, the Warren Oyster Company in 1952 ceased culture operations, were all of the old oyster leases eventually revoked. The creation of a Coastal Resources Management Council in the late 1970s and changes in the aquaculture laws (General Laws of Rhode Island 20-10-1) in the early 1980s included provisions against aquaculturists retaining leases in public trust waters when active aquaculture operations cease (Olsen and Seavy 1983).

Throughout the 1950s and 1960s, there were countless discussions about how the quahog fishery should be managed. The main issue was whether the fishery would consist of a large number of small-scale operators using hand tongs and bullrakes or a relatively small number of operators using power dredges. The rakers and tongers argued strongly to management officials that power dredging was environmentally damaging. This prompted a number of studies, including that of Glude and Landers (1953) which showed that while dredging did allow individuals to harvest more shellfish in a shorter period of time, it was no more damaging than the cumulative impacts of large numbers of handrakers. By the early 1960s, state management officials set into statute the banning of power dredges in most of Narragansett Bay (General Laws of Rhode Island 20-6-7). Thus the strategy was to allow greater numbers of fishermen through limits on individual effort. Under this system of limiting individual effort, the fishery grew and flourished. When the Rhode Island quahog fishery reached its peak in 1985, there were an estimated 1000 full-time commercial shellfishermen, landing 2200 metric tonnes (meat weight) of shellfish, worth $15 million dockside, representing about 25% of all quahog production nationally (Boyd 1991; Pratt et al. 1992).

During the decade of the 1990s there has been a steady decline in quahog catches and a decrease in the number of active full-time shellfishermen. According to the Rhode Island Department of Environmental Management statistics in 1997, there was an estimated 500 full-time fishermen landing about 651 metric tonnes (meat weight) of shellfish. Rhode Island's national market share in quahogs has dipped to about 8%. This erosion of the fishery since the 1980s has caused concern in the industry and generated calls for means to rebuild the fishery through expanded relays, seeding, and other public aquaculture projects.

OVERVIEW

Concern about declining shellfisheries is certainly not new in Rhode Island. Through the years there have been numerous attempts to use aquaculture techniques to enhance fisheries. As previously noted, oysters were transplanted onto lease beds for maturation, but this was more of a directed private enterprise practice

on privately held lease beds. The first genuine public aquaculture for a marine species was a lobster hatchery established in Wickford Harbor in 1898 by the Rhode Island Inland Fisheries Commission in response to declining lobster fisheries (Meade 1901). As one contemporary account put it, "It is no exaggeration to say that in practically every known natural region of the North Atlantic coast, the lobster fishery is either depleted or in a state of decline" (Herrick 1909). Personnel from the hatchery would gather berried female lobsters from the Narragansett Bay trap fishery, and carefully incubate the eggs until hatching. They maintained larvae in floating fine mesh net cages and fed them a diet consisting of ground beef liver and cooked chicken eggs. While in the floating cages, larvae were gently agitated with a mechanical apparatus to keep them suspended to reduce cannibalism (Meade 1908). After reaching fourth or fifth postlarval stage, they were released into Narragansett Bay. In the first rear of lobster releases, 1901, only 9000 juveniles were released into Narragansett Bay. But in 1908, the Wickford station was releasing 400,000 lobsters, and by 1920 the facility was releasing over 1 million lobsters yearly. Peak production of the facility was in 1934 when lobster releases reached over 1.5 million (IFC 1934). The lobster hatchery program continued by the Inland Fisheries Commission (IFC) until 1935, when the agency was reorganized into the Department of Fish and Wildlife. The Wickford lobster hatchery continued under Fish and Wildlife until the 1940s (Carlson 1954). After nearly a half century of operation this remarkable pioneer program was eventually discontinued as a cost cutting measure. The site of this first state lobster hatchery is now used by the Rhode Island Department of Environmental Management's Division of Fish and Wildlife as a fisheries laboratory and a base for the patrol craft of state fisheries conservation officers.

The first Rhode Island oyster hatchery was established by Paul Galtsoff in the late 1930s at the end of South Ferry Road in Narragansett, on what is now the campus of the University of Rhode Island's Graduate School of Oceanography (GSO). The intent was to establish a facility similar to the successful oyster hatchery in Milford, Connecticut, operated by Victor Loosanoff of the Bureau of Commercial Fisheries (the forerunner of the National Marine Fisheries Service) to aid the Long Island Sound oyster industry. Galtsoff operated the hatchery for a time but it had little impact on the then moribund Rhode Island oyster industry; however, it became the forerunner of the National Marine Fisheries Service Narragansett Laboratory. The hatchery building itself remains on the GSO campus as the Mosby Center, the campus cafeteria building. The other legacy of the period is Galstoff's valuable reprint collection used as reference material in his classic (1964) treatise on the American oyster. The collection is housed currently at the nearby Pell Marine Science Laboratory.

As the quahog fisheries began to develop in the late 1940s and early 1950s, much of the quahog stocks in Narragansett Bay were located behind pollution closure lines. The first baywide wide assessment of quahogs in Narragansett Bay showed that greater than 60% of the quahogs in the bay resided in the closed Providence River and Mount Hope Bay sections of the bay (Stringer 1959). There was intense fishing pressure on the remaining open areas by both hand-diggers and dredge boats. In 1954, the Division of Fish and Wildlife initiated a shellfish relay program to dredge quahogs from the closed areas and deposit them in management areas in certified waters that would eventually be open for fishing. Between 1954 and 1968, the relay program typically moved an average of 1140 metric tonnes of shellfish annually (Table 1).

TABLE 1.

Quahog relays in Narragansett Bay in metric tonnes whole shell weight (Source: Division of Fish and Wildlife Annual Reports).

	Method of Harvest		
Year	State Dredge Vessels	Hired Dredge Boats	Handraking
1954	—	5774	—
1955	—	4697	—
1956	5163	1725	—
1957	4998	—	—
1958	4374	2767	—
1959	4695	—	—
1960	3125	—	54.4
1961	2932	—	20.4
1962	4027	820	138
1963	1169	—	83.6
1964	—	7016	—
1965	—	7487	361
1966	—	7702	361
1967	—	6412	—
1968	—	2916	99.2
1971	329	—	—
1977	—	—	66.9
1978	—	—	85.7
1979	—	—	71.8
1980	—	—	86.5
1981	—	—	87.7
1982	—	—	81.6
1983	—	—	49.9
1984	—	—	108.2
1985	—	—	59.8
1986	—	—	103.4
1987	—	—	88.5
1988	—	—	133.5
1989	—	—	101.7
1990	—	—	322.4
1991	—	—	25.1
1992	—	—	108.1
1993	—	—	7.0
1994	—	—	78.3
1995	—	—	7.5
1996	—	—	60.3
1997	—	—	122.2
1998	238	—	290.5

After 1968 and the banning of power dredging in Narragansett Bay, the transplant program changed character. Beginning in 1977, under the leadership of Arthur Ganz, the state-conducted relay program began paying a modest fee to supervised shellfishermen to dig quahogs and move them to the management areas in certified waters. On average over 22 y, 98 metric tonnes of shellfish were moved annually (whole shell weight; ranging from 7 to 322 metric tonnes per year). This program remains popular with the shellfishing community and has been ongoing until the present.

In spite of their popularity in the shellfishing community, the value of relay programs has long been in dispute in Rhode Island. The reluctance by state officials to dedicate permanent funding for a long-term shellfish transplant program has led to intermittent stocking attempts, primarily into already highly productive areas such as Greenwich Bay. Critics have classified the program as a "put & take" subsidized fishery, while shellfishermen contend they are denied access to highly productive areas due to long standing

sewage treatment deficiencies and argue for just compensation. Since the 1980s, shellfishermen have become dependant upon the Greenwich Bay transplant area for economic survival during the winter months. While a limited number of shellfishermen traditionally participate in transplants, hundreds of fishermen are observed harvesting upon the reopening of management areas.

Another effort to restore shellfisheries in Rhode Island included an effort by the Shellfish Commission of the Town of New Shoreham (Block Island) between 1987 and 1990 to rear hatchery seed quahogs in plastic-mesh-covered trays (Littlefield 1991). In 1989, about 120,000 *Mercenaria mercenaria* (*notata* strain) 15–20 mm in size were seeded into the Andy's Way section of the Great Salt Pond. In 1990, about 130,000 more were seeded into the same area. Littlefield (1991) reported that legal-size (about 48 mm valve length) *notata* quahogs were showing up in the 1990 fall fishery, but he did not estimate a percentage recovery rate. This project was discontinued in 1991 when Mr. Littlefield resigned from town government.

In addition to quahogs, scallops have been an important shellfishery in Rhode Island. Beginning in the early 1970s there were three major pushes to restore scallop (*Argopecten irradians irradians*) fisheries using hatchery reared seed. Scallop fisheries were historically abundant in the shallow Greenwich Bay region of Narragansett Bay and the barrier beach lagoons and estuaries along the south shore of the state (Olsen and Stevenson 1975). By the late 1960s and early 1970s, scallop catches were small and sporadic. As a result the Division of Fish and Wildlife established a hatchery for scallops at the old ferry terminal in Jamestown in 1973. The operation was moved to better facilities at the Division of Fish and Wildlife Coastal Fisheries Laboratory in Jerusalem in 1978. During its 7 y of operation, several thousand scallops were distributed to coastal barrier beach ponds. The hatchery ceased operations in 1980 for lack of continued state funding (John Karlsson, Division of Fish and Wildlife retired, personal communication 1998).

The next scallop restoration effort, in 1988–1991, was the Rhode Island Scallop Restoration Project. This effort arose in to response to massive scallop and other bivalve mortalities as a result of blooms of the picoplankton *Aureococcus anophageferens* in Narragansett Bay and coastal estuaries during the summers of 1985 and 1986 (Tracey 1988). This 'brown tide' event occurring on two successive years decimated scallop (*Argopecten irradians irradians*) populations in the coastal salt ponds and estuaries. The aim of the restoration project was to solicit funds as a nonprofit corporation and to work cooperatively with the Division of Fish and Wildlife and Spatco Ltd., a private hatchery, to hatch and rear juvenile scallops for release in the coastal ponds (Burns 1991). As a result of this collaborative project, 60,000 seed scallops (15–20 mm size) were released into Point Judith Pond in 1989. In 1990, 20,000 seed (15–20 mm) were released in the Great Salt Pond of Block Island, and 5 million seed (1–3 mm) were released into Point Judith Pond due to lack of adequate nursery facilities. Also that year, 250,000 seed (15–20 mm) purchased from a Maine hatchery were distributed to Quonochontaug, Ninigret, and Winnepaug ponds (Robert Rheault, Spatco Ltd., personal communication, 1998; Division of Fish and Wildlife records). The project was discontinued in 1991 because the nonprofit corporation could not raise sufficient funds to continue, and the intricacies of state bid-procurement regulations made advance seed orders from the private hatchery participating in the project a challenging process.

CURRENT SHELLFISH RESTORATION

Scallop

The third scallop restoration effort, the Restoration and Enhancement of Bay Scallop Populations in Narragansett Bay Project, has been underway since 1995 through the Rhode Island Department of Environmental Management Aqua Fund. The aim was to reintroduce bay scallops, *Argopecten irradians*, to areas of historical abundance in Narragansett Bay, using both hatchery-reared scallops and scallops gathered from mesh spat collector bags similar to those recently used for scallop restoration in nearby Westport, Massachusetts (Tammi et al. 1998). Seed averaging 20 mm (range 15–24 mm) was both free planted and placed in protective cages for overwintering at sites with suitable habitat. The scallops were monitored for growth, survival, and predation rates. A total of 1 million seed had been planted in Narragansett Bay as of Fall 1998, producing small sets of bay scallops in adjacent areas of some of the sites. While overall recovery of planted scallops has been minimal, several areas of the bay supported a recreational harvest of bay scallops for the first time in decades.

In 1997, additional funding was received to add scallop stocking of the south shore coastal ponds, utilizing the same methodology as the Narragansett Bay study. The coastal pond study provided control sites for comparison with the existing sites in the bay. A total of 80,000 hatchery-reared scallops were planted in the fall of 1997 and again in 1998. Growth and survival rates of scallops placed in the coastal ponds were significantly higher than at any of the sites in Narragansett Bay, despite observations of equally high predation and fouling. Additionally, populations of seed scallops have been observed in the vicinity of caged animals, as well as being found in two of the five ponds where spat collectors were deployed. The project is in its final year of monitoring and as of December 1998 there is no dedicated funding on the horizon to continue scallop enhancement efforts.

Lobster Fishery

After nearly a 50-y hiatus, there has been a revival of the idea of restoring and enhancing Narragansett Bay lobster fisheries beginning in 1996 in a cooperative study between the University of Rhode Island and the National Marine Fisheries Service (Cobb et al. 1998). Funded by restoration and remediation funds from a 1989 oil spill in Narragansett Bay, the aim of this modern effort is to increase lobster habitat, assess the survival of lobsters naturally recruiting onto artificial reefs, and assess the survival of hatchery-reared lobsters on the artificial reefs. This approach of focusing on habitat differs from the earlier approach of releasing juveniles directly into the bay without protective habitat. Six artificial reefs (10 × 20 m) consisting of either cobbles or boulders were placed in Narragansett Bay. Soft bottom and naturally rocky bottom control areas served as control areas. In 1996 and 1997, natural lobster recruitment into the areas was monitored. In 1998, about 2400 fifth stage, tagged juvenile lobsters (4.3/m^2) were released into the test sites (Kathleen Castro, University of Rhode Island, personal communication 1998). This enhancement program should run until 2001 and yield valuable data about habitat enhancement as a means for reducing predation on released hatchery-reared stock.

Quahog

In response to declining quahog fisheries in the 1990s, the Rhode Island Department of Environmental Management (DEM),

Division of Fish & Wildlife (DFW) began a population and utilization study of the uncertified shellfish resources in the Providence River (funded by a grant from the U.S Department of Commerce). Evaluating shellfish relay programs as a tool for enhancing Rhode Island's quahog fishery is essential to the development of a statewide shellfish management program. Judicious utilization of shellfish resources in uncertified waters for either relay stocking or depuration may provide revitalization of Rhode Island's shellfish industry. Project activities included a survey to determine current quahog biomass in the Providence River, calculation of estimates of maximum sustainable yields, and development of a rational plan for the transplanting of uncertified shellfish stocks. One of the key work elements of the project was a pilot project to test the feasibility of transplants. The DFW is evaluating different methodologies of transplanting for optimum benefit for the resource and the industry.

During 1997, DFW conducted shellfish dredge surveys in the Providence River to assess quahog population densities. This area had not been surveyed since 1977. The survey was conducted from onboard the 29′ R/V *Inspector Clambeaux* utilizing the same random stratified procedures pioneered in a DFW study of quahog populations in the West Passage of Narragansett Bay (Russell 1972). These techniques have been used annually since 1993 for assessing quahog populations in other areas of Narragansett Bay (Lazar et al. 1995). The Providence River stations were divided into 500 × 500 m grids and a 30-m tow was made using a hydraulic dredge. From these samples the DFW determined quahog abundance, size structure, and densities, and the maximum sustainable yield (MSY) that could be transplanted without depleting the stock. The total of 51 tows completed had a mean density of 9.37 ± (1.34 se) quahogs/m^2. Total standing stock biomass for the Providence River was 26,400 metric tonnes. The biomass was 86% top necks (60 mm valve length or larger); few sublegal or count necks were observed over the course of the survey. Subsamples of the quahogs were measured and morphometric comparisons calculated between shellfish in certified and uncertified waters. A baywide MSY was calculated using a biomass dynamic model that integrates catch per unit effort (CPUE), landings, and survey data. Data analysis indicated a maximum of 2721 metric tonnes could be removed annually from the Providence River for relay purposes without impairing stock production. For year 1 (1998), DFW recommended a minimum of 238 metric tonnes be harvested by dredge vessel for transplanting during this prototype project.

Prior to commencement of the transplant, several areas were evaluated for appropriate bottom types, existing shellfish densities and current fishing effort, predation, and proximity to depleted areas. The "High Banks" Shellfish Sanctuary was established as a management area and closed to commercial harvesting of shellfish for a period of 2 y (Fig. 1). The expectation is that this dense concentration of large adult clams will repopulate adjacent areas. The RV *Captain Bert* from the University of Rhode Island was contracted to dredge from July to November 1998. A total of 238 metric tonnes of shellfish were moved from the Providence River; all tow positions were logged using global positioning system (GPS) coordinates. Data collected onboard included tow coordinates, depth, bottom type, density, and shell measurement. These data will be utilized to update the 1997 biomass assessment. Additionally, the donor area and the adjacent highly productive "Area A" (or Upper Narragansett Bay area) will be monitored through annual dredge surveys to provide estimates of the impacts of the large-scale relays and of quahog recruitment in those areas. The

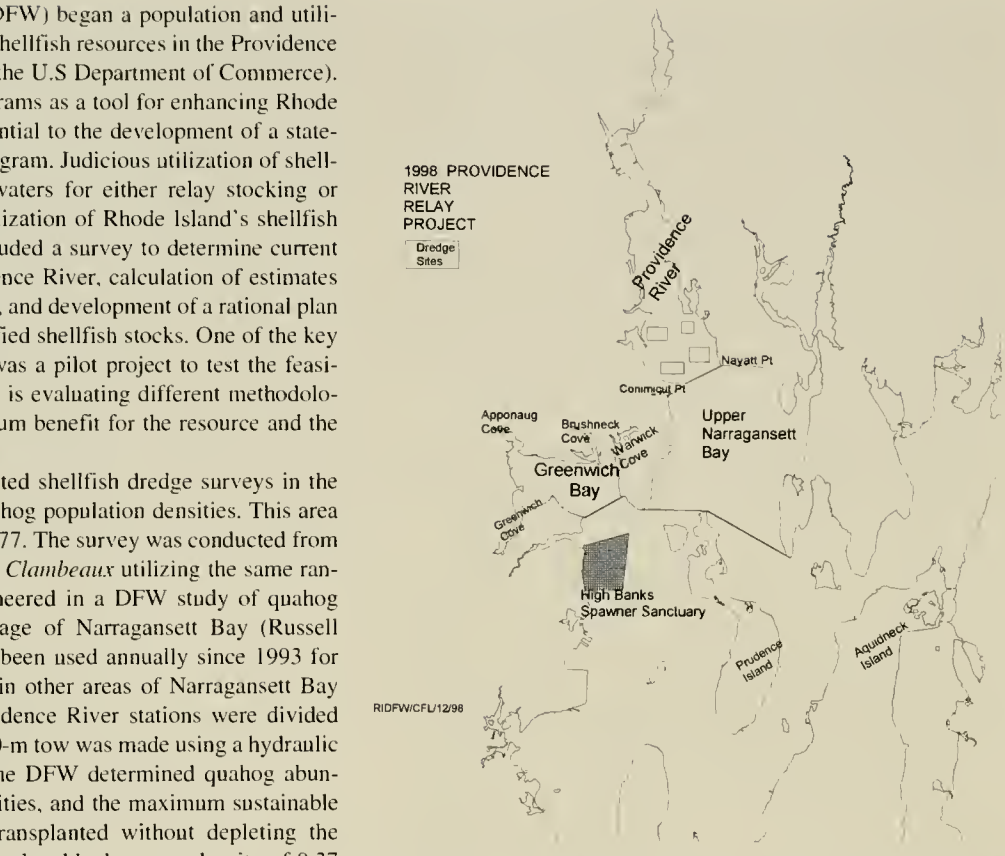

Figure 1. The source area of quahogs for the 1998 Providence River Relay Project and the recipient High Banks Sanctuary Area in Narragansett Bay Rhode Island. Also shown are the shallow coves of Greenwich Bay that serve as the source areas of quahogs for handraking relays into the Greenwich Bay Management Area.

High Banks relay area will also be monitored over 1999–2000 to address the efficacy of large-scale relays of the Providence River stock. To address recovery, growth, predation, fishing pressure, and mortality, a portion of the relayed stock has been tagged and their location logged by GPS coordinates. They will be monitored by SCUBA and DFW's dredge research vessel in 2000.

Cost analysis of the dredge relay program indicates $0.09/kg was expended to move shellfish, compared to a range of $0.08 to $0.13/kg typically expended by contracting handrakers. Expected costs for utilizing handrakers in the Providence River would likely have been well in excess of $0.13/kg, due to the added expense of daily contracting of transport vessels and the additional personnel costs for monitoring and enforcement. Also, logistically it is unworkable to provide adequate supervision of individual rakers in a relatively large area in the midst of a major shipping channel. However, contracting handrakers to move shellfish from smaller coves immediately adjacent to recipient areas appears to be an economical option, as is the case for the current Greenwich Bay relay program.

In addition to the relay efforts, there has been interest in using nursery-reared quahog seed stock for replenishing public fishing grounds. In 1996, John Williams of Warwick Cove Marina seized upon the idea of nursery culture of shellfish in marina waters (Rheault and Rice 1989) using existing floating upweller techniques (Hadley & Manzi 1984) being developed and employed by Robert Rheault of the Moonstone Oyster Company. At the incep-

tion of the Rhode Island Public Benefit Aquaculture Project, Mr. Williams' concept was to incorporate secondary education into the physical activities of raising shellfish. The project would be a means of teaching science, math, and language arts, and the product would be used to restore fishing beds in Narragansett Bay. The project, which began in 1997 with some seed monies from local foundations, had as its a mission to develop a true public benefit aquaculture project that would integrate well with current uses of the public waters of Rhode Island, without compromising the integrity or quality of the state's aquatic resources. The Rhode Island Seafood Council began assisting in the development and coordination of a team to guide this effort. The Project team realized early on that in order to be fully beneficial to the state, all stakeholders needed to be educated about the project goals.

The Project goals include:

- Strong skills-based curriculum development for secondary level education that is tied to natural resources, and the application of aquaculture principles to inject applied learning into the curriculum.
- The inclusion of commercial and recreational shellfishermen in order to build their understanding of the potential of public aquaculture to rebuild shellfish stocks, and to utilize their experience in guiding educational activities and assisting in the determination of survival of seeded stock
- The development of a self-sustaining resource restoration plan for Rhode Island incorporating aquaculture techniques to restore declining shellfish resources.

To meet the goals, a strong team of state, local, and educational representatives committed to guide the development of the Project. Initial advisors to the project were university personnel with expertise on scientific issues and independent commercial fishermen and personnel from marine trades organizations to assist in developing an economically viable plan of work that minimized user conflicts. The advisory team grew to include representatives from the following organizations:

Rhode Island Department of Labor and Training
Rhode Island Manufacturers Extension Service
Rhode Island Legislative Commission on Aquaculture
Rhode Island Economic Development Corporation
Rhode Island Department of Environmental Management
Coastal Resources Management Council
Rhode Island Seafood Council
University of Rhode Island-Department of Fisheries and Veterinary Science
Roger Williams University Center for Economic and Environmental Development
Newport, Warwick, Chariho, and Cranston Area Career and Technical Centers
Middletown Alternative Learning Program
Rhode Island Quahog Company
Warwick Cove Marina
Ram Point Marina
East Passage Yachting Center
Newport Yacht Club
The Rhode Island Shellfishermen's Association

Students from four technical high schools began building upwellers in the spring of 1998. These schools were partnered with marinas in the local area, and high school students were partnered with college-level mentors from the Marine Biology Program at Roger Williams University in Bristol. Three of the high schools built five upweller units that were deployed early summer at partner marina facilities. The five units were stocked with a total of 100,000 seed of *Mercenaria mercenaria*. Due to an unusually high rainfall in the spring, quahog seed was very difficult to obtain from local suppliers. As a result, quahog seed (2.5 mm) was purchased from Virginia after satisfying strict state importation guidelines. In addition, one unit was stocked with local oyster seed donated by the Rhode Island Sea Grant Marine Advisory Service and another unit was partially stocked with scallop seed donated by National Marine Fisheries Service Milford Laboratory.

As the quahog seed reached the presumed "predator resistant" size of 20 mm they were given to the DFW and planted in a management sanctuary had been opened for public harvest in December 1999. Current Rhode Island shellfish regulations define shellfish seed as a shell dimension of 20 mm or less for quahogs and 32 mm for oysters (RIDEM 1998). To assure public health, shellfish seed can be grown in uncertified waters of marinas, but they must be moved to certified waters for final growout and depuration once they reach the prescribed size limits. These seed definitions allow a minimum 1-y depuration period based on local growth rates.

Shellfish growth was monitored weekly by students from the partner schools and all data on growth, salinity, and temperature were recorded. As the shellfish grew students were able to apply math, science, writing, and public speaking skills to the project through presentations at their respective schools and at the Third Annual Rhode Island Aquaculture Conference, held in October 1998. This was a perfect way for students to start building a portfolio and to create a network of professional mentors. After the conference, there have been several other schools and marinas that expressed interest in participating in the project.

This collaboration has also allowed students to work alongside state biologists and to be involved in data collection for the tagging and transplant-restoration project at the High Banks Management Area being carried out by DFW. All students that have taken part in the tagging and relay-restoration effort have a better understanding of occupations within DFW, and have more direction in their education and future career choices. Additional statewide partnerships are being built: the Rhode Island Department of Health has become eager to expose students to the workings of a USFDA-certified shellfish testing lab and is making the lab available for interactive tours for the students involved in the aquaculture program.

As of December 1999 there are two proposed plans with potential to assist ongoing shellfish restoration projects in Rhode Island. One proposal is to start a commercial shellfish hatchery by the Hope Shellfish Company LLC at the Quonset Point Industrial Park, which would be partially funded from private investors, the Rhode Island Economic Development Corporation, and the Rhode Island Economic Policy Council. Part of the stated mission of the proposed hatchery is to provide shellfish seed for public aquaculture and mitigation projects in the state. The other proposal is a plan to mitigate damages caused by the January 1996 grounding of the barge *North Cape* and the devastating oil spill that resulted on the south shore beaches of Rhode Island. The National Marine Fisheries Service in cooperation with Rhode Island state agencies is proposing an ambitious multi-year plan to re-seed molluscan shellfish beds in the coastal barrier beach salt ponds and estuaries near the spill site.

CONCLUSIONS

Shellfish restoration efforts in Rhode Island have a long history. It is very instructive to examine past projects to discover the

reasons for either success or failure, and to apply these lessons to current and planned efforts. There are a number of attempted projects and a number that got started but did not prove to be sustainable in the long run. Some of the projects that have arisen out of a sudden "windfall" of funds or out of short-term public concern have been particularly susceptible to not building long-term sustainability. Some projects have flourished due to individual initiative, but failed to continue when the principals either "got tired" or moved from the area. The most successful projects in terms of longevity are those that have had perennial support by commercial and recreational fisheries clientele, local communities, and state government agencies. For successful shellfish restoration projects, there must be melding of good science, consensus on policy, public acceptance, economic feasibility, multisector cooperation, and a measure of good luck.

A cautionary note, however, is needed. In recent years there has been an increasing trend in academia and government toward collaborative, multi-agency, and multisector projects for greater cost effectiveness, sharing of talent, and sharing of resources. Although the potential benefits of collaborative multisector projects are great, there are some risks. It is short-sighted to underestimate the amount of time and effort required to bring people and organizations together and to maintain continued collaboration toward a common goal. This problem of coordination and management is not intractable; it simply needs to be recognized and planned for.

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A PLAN FOR REBUILDING STOCKS OF OLYMPIA OYSTERS (*OSTREOLA CONCHAPHILA*, CARPENTER 1857) IN WASHINGTON STATE

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ABSTRACT The Olympia oyster (*Ostreola conchaphila*) is native to the state of Washington. Once the basis for a thriving, statewide oyster industry, its numbers were drastically reduced by the mid 1940s. Water quality and overharvesting are thought to be the major factors causing its near demise. The Pacific oyster (*Crassostrea gigas*) has since replaced the Olympia oyster in Washington and world markets. Concern over the much reduced status of the stocks of native oysters led the Washington Department of Fish and Wildlife to develop a plan to rebuild them. The goal of the strategy, to restore the Olympia oyster within its historical range, is quite simple, but offers many challenges. Key elements of the draft strategy presented here include a description of the historical and current distribution, habitat requirements, and current problems associated with restoring stocks of the native oyster in Washington State. Partnering with Tribal co-managers, local commercial shellfish interests, and the general public provides new opportunities for restoring the Olympia oyster, a top priority for state management of this species. Suggested priorities for strategy implementation are also discussed.

KEY WORDS: Olympia oyster, *Ostreola conchaphila*, Washington, estuary, plan

INTRODUCTION

The Olympia oyster (*Ostreola conchaphila* Carpenter, 1857; formerly *Ostrea lurida*) is native to the Pacific coast of North America and occurs in marine waters from Bahia de San Quentin, Baja California, to Sitka, Alaska (Ricketts and Calvin 1968, Baker 1995). It primarily inhabits sheltered waters or estuaries. Once common in Washington state, the Olympia oyster now has a restricted and very patchy distribution in Willapa Bay, Grays Harbor, and southern Puget Sound.

The Olympia oyster has been the focus of human harvest for several thousand years. The Washington tribes used the oyster extensively and often based settlement locations on its harvest (Swan 1857, Washington Secretary of State 1935, Steele 1957, Elmendorf and Kroeber 1992). With European colonization, the Olympia oyster supported a large commercial industry. Olympia oyster beds in Puget Sound, Hood Canal, and Willapa Bay were harvested extensively, and later cultivated with an elaborate system of dikes (Steele 1957, Brown 1976, Westley et al. 1985). Overharvesting in the late 1800s and severe water quality problems in the 1930s to 1950s caused Olympia oyster stocks to crash, and the industry to terminate in Willapa Bay and Puget Sound, respectively.

Recent interest and concerns about the status of native Olympia oyster stocks in Washington State waters, widely recognized to be reduced from historical levels, led the Washington Department of Fish and Wildlife (WDFW) to begin developing a stock rebuilding plan. General goals of the plan are wise stewardship, maintenance of genetic integrity, and ecosystem restoration. As managers, however, we struggled with the scope of the goal statement, particularly whether or not to incorporate restoration to a level where fisheries could occur. The resulting, more generic statement we accepted, "*to restore and maintain Olympia oyster populations on public tidelands in their native range.*" defers that discussion to a later date.

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STATUS OF THE POPULATION

Historical Population Size and Distribution

Historically, Olympia oyster stocks were very large in Washington State, with reported annual landings of over 130,000 bushels (4581 m^3) around 1890, principally from Willapa Bay (Townsend 1896, Fig. 1). Landings were substantially higher in the 1870s (possibly as high as 200,000 bushels), but landings were not well documented. Olympia oysters are very small so this represents a substantially greater number of individuals (approximately 1500 per bushel) than are found in current harvests of *Crassostrea gigas* (Thunberg 1793).

Within the inland waters of Washington, the Olympia oyster had a scattered distribution throughout Puget Sound and Hood Canal (Westley 1976, Fig. 1). Samish Bay once supported a large, naturally occurring Olympia oyster population, which was severely depleted in the 1800s as a result of overharvest (Brown 1976). The most abundant natural Puget Sound populations historically occurred around Olympia, primarily in Mud and Oyster bays (Steele 1957).

Current Population Size and Distribution

Native oysters are currently present on state oyster reserves in Willapa Bay on the coast, and North Bay and Case Inlet in southern Puget Sound. Dense natural sets have recently been observed in the reserves in southern Puget Sound and an annual spawning event has been noted in Willapa Bay, but very few juvenile oysters

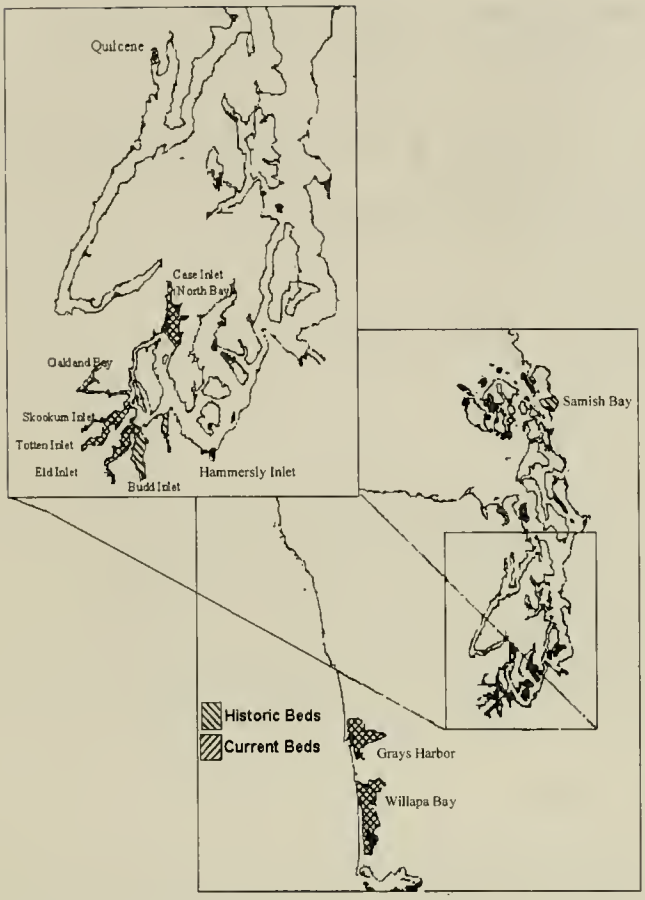

Figure 1. Map of Washington state showing the general distribution of historic and current Olympia oyster (*Ostreola concaphila*) stocks.

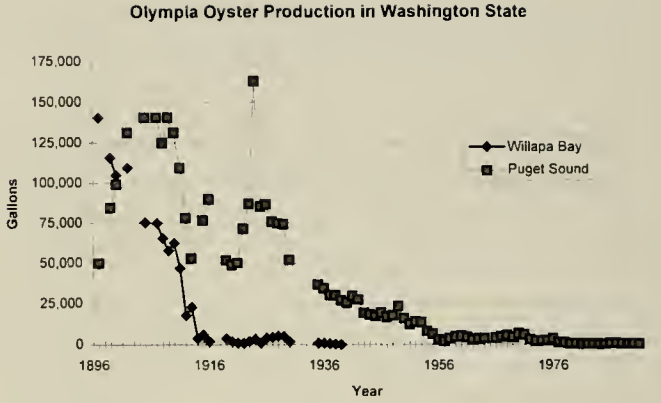

Figure 2. Olympia oyster production in Willapa Bay along the outer coast and Puget Sound in Washington State. Production in Willapa Bay declined rapidly in the early 1900s while that in Puget Sound remained higher through the mid 1930s. Production in Willapa Bay prior to 1896 was likely higher, but records are difficult to locate.

appear to have survived at the latter location. Shellfish growers in south Puget Sound had noticed similar sets in the mid 1980s, but these sets were subsequently destroyed by severe winter weather. Comparison of historical documents and local knowledge indicates that current numbers are, at best, a mere fraction of, and possibly more ephemeral than, historic populations.

Management

The Washington Department of Fish and Wildlife (WDFW) co-manages Olympia oysters with the Tribes and other government agencies in Washington state according to the provisions in the federal district court's orders and judgments (*United States v. Washington*, No. 9213, subproceeding 89-3).

Non-Tribal Commercial Fishery

WDFW harvest reports from 1897 through 1990 (Fig. 2) show a general decline in commercial production of Olympia oysters, from a high of over 200,000 gallons (757,072 L) in the early part of the century to an annual production of less than a 1000 gallons (3785 L) since 1979. Preliminary data for 1991 through 1996 show an annual commercial harvest of approximately 500 gallons (about 4000 pounds = 1814 kg of shucked oysters), most of which is from private tidelands in south Puget Sound.

Currently there are three commercial Olympia oyster growers in Puget Sound that operate solely on private tideland. The private grower controls management of stocks on private tidelands; however, Washington State Department of Health requirements must be met, and quarterly harvest reports are required by WDFW.

The Washington state oyster reserves were established in 1890 for the preservation and growth of Olympia oysters (Woelke 1969). Reserves were set aside to provide both "seed for the oyster farmer and an exploitable stock for the fisherman." At the time these laws were enacted, the typical practice in Willapa Bay was to tong or handpick the native oysters from low intertidal areas and move them to privately held tidelands elsewhere in the bay. They were held until they were shipped to markets in San Francisco, California, and Portland, Oregon. The reserves originally comprised 4548 ha in Willapa Bay and 1821 ha in Puget Sound. Some lands have since been sold by the state legislature. Reserves currently encompass 4047 ha in Willapa Bay and 405 ha in southern Puget Sound.

With the decline in the Olympia oyster population, reserve laws were changed in 1947 to reflect the growing importance of the Pacific oyster, *Crassostrea gigas*, which was imported into Washington state in the mid 1920s and rapidly established itself on reserve tidelands, particularly in Willapa Bay. Willapa Bay reserves are now actively managed for commercial harvest of the Pacific oyster. An average of 54,000 bushels (1903 m^3) of Pacific oysters are sold each year from managed intertidal tracts, as a result of which $79,000 is returned annually to the state general fund (Dumbauld and Kauffman 1996). Growers are required to return 40% of the live oyster volume in shell to the tracts to maintain stocks via natural spawning and settlement.

No commercial oyster harvest occurs on the Puget Sound oyster reserves. While Olympia oysters exist on both the Willapa Bay and Puget Sound reserves, no active management has occurred for this species, and the last commercial harvest of Olympia oysters on reserve tidelands occurred in Puget Sound in 1929.

Non-Tribal Recreational Fishery

The Olympia oyster has been managed passively on public beaches in Washington state for many years. Olympia oysters are included in the regulations that apply to all classified oysters. Current harvest limits include a combined daily limit of 18, and oysters must be shucked on the beach and the shells left at the

same place and tide height where they were taken. They may be harvested only by hand or with a hand-held manually operated prying tool (no hammers, etc.). Beginning May 1, 1998, regulations were changed for all areas except Hood Canal and the outer coast. These regulations included a minimum size restriction of 6.35 cm (2.5 inches), which was designed to minimize recreational harvesting of Olympia oysters.

All oyster reserves continue to be closed to recreational harvest of intertidal clams and oysters with several exceptions where clams and Pacific oysters may be taken. Seasons for non-reserve beaches are set based on the population and projected harvest of Pacific oysters. With the exception of one beach in North Bay, Puget Sound oyster reserves currently are not actively managed for oysters. Oyster dikes in Oakland Bay, once designed for Olympia oysters, have created excellent habitat for Manila clams (*Tapes phillipinarum*, Adams & Reave, 1850). Although the majority of the Puget Sound reserves are closed to recreational clam and oyster harvest due to access issues, these Manila clam beds do provide stock to trade with the tribes to enhance other recreational opportunities.

Tribal Commercial Fisheries

The tribes of inland and coastal Washington historically have played a dominant role in the commercial harvest of Olympia oysters (Steele 1957) and at least one tribal war was fought over rights to harvest Olympia oysters (Swan 1857, Esveldt 1948, Steele 1957). There are no current Tribal commercial fisheries targeted for Olympia oysters. However, commercial harvest of Olympia oysters is not prohibited in the State/Tribe Interim Management Agreement except in areas the state has declared as artificial beds. The Point No Point Treaty Council Tribes are the only tribes that have issued regulations for the commercial harvest of oysters (species not specified) on public tidelands. Their annual commercial regulations have a clause prohibiting the harvest of oysters less than 6.35 cm in length for single oysters, which would eliminate virtually all harvest of Olympia oysters. The majority of tribal oyster bed openings are for single oyster harvest, but some harvest of Olympia oysters could occur when clusters are harvested, which has occurred recently at a few beaches. Olympia oysters may be harvested in ceremonial and subsistence fisheries.

Genetic Integrity of Olympia Oyster Stocks

Research suggests that the rate of natural genetic exchange is low among distinct coastal populations of native oysters in Washington, Oregon, and northern California (Baker 1995); however, no information exists on genetic exchange within Washington waters. This is particularly important when considering historic Olympia oyster farming practices, which included seed transport both within and between regions.

Conserving the natural genetic integrity of Olympia oyster stocks is an important component of the stock recovery strategy. Artificial enhancement of Olympia oyster stocks should meet acceptable standards for maintaining the genetic stock integrity for indigenous species. These standards include (1) using brood stock for seed production from the same geographic area where seeding will take place, and (2) establishing and maintaining the minimum number of brood stock necessary to maintain genetic variability while maintaining stock identity. Genetic integrity will be a topic for further dialog in efforts to rebuild Olympia oyster populations.

FACTORS AFFECTING THE POPULATION

Habitat and Water Quality

Pollution has been the primary factor in the demise of the Olympia oyster throughout lower Puget Sound and Hood Canal. Sulfur waste liquor (SWL) from the Rayonier pulp mill, which was built on Oakland Bay in 1927, was identified as the cause of the demise of all Olympia oyster stocks in south Puget Sound (Gunter and McKee 1960). Tidal currents carried effluent to Oakland Bay beds within a tidal cycle and throughout lower Puget Sound within a matter of days. Dramatic crashes were witnessed throughout the Olympia oyster beds, and the Olympia oyster industry was destroyed by the mid 1940s. The Rayonier mill was closed in 1957. Unfortunately, monitoring of the Olympia oyster populations of Puget Sound and southern Hood Canal had ceased following the crash (Steele 1957, Gunter and McKee 1960).

Water quality impacts in Washington's waters have shifted over the last 40 years from those caused by point source industrial effluent to nonpoint source pollution. The impacts of contemporary water quality degradation to residual Olympia oyster stocks have not been studied. Possible contemporary sources of pollution and water quality impacts affecting Olympia oysters include low dissolved oxygen (DO), chlorine from sewage outfalls, nonpoint pollution and associated eutrophication, sedimentation and siltation, and herbicides (McMillen 1978, Couch and Hassler 1989, Dumbauld 1996).

Harvest

Overharvesting has been identified as the leading cause of Olympia oyster stock crashes in Samish Bay (Puget Sound) and Willapa Bay in the 1800s. Harvesting of other commercially valued species may also impact Olympia oysters where they co-occur.

Interspecific Interactions

After the initial population declines, additional factors have contributed to preventing the recovery of Olympia oysters. Introduced predators (the Japanese oyster drill *Cerateotoma inornatum* Reduz, 1851, flatworms *Pseudostylochus ostreaophagus*,Hyman, 1955, and the copepod *Mytilicola orientalis*, Mori, 1935) have resulted in poor oyster conditions, and in the case of drills have caused high mortalities (Peters 1993). Natural predators, including starfish and diving ducks, are also thought to suppress recovery of Olympia oyster populations. Other disturbances, including substrate disruption by ghost shrimp and mud shrimp, smothering by slipper shells, and competition with Pacific oysters for space and setting habitat, are suspected of negatively affecting Olympia oyster recovery (Steele 1957, Brown 1976, Westley 1976, Dumbauld pers. comm.).

STOCK REBUILDING ACTIONS

Restoration of this species may include both natural and artificial enhancement strategies. Natural restoration techniques, such as water quality and habitat improvements will be the primary focus. Primary objectives and actions necessary to rebuild Olympia oyster stocks in Washington state vary with region but include:

(1). Working with local experts, including the Tribes and shellfish growers, to define the historic and current distribution of the Olympia oyster.

(2). Conducting population surveys to define current population levels and establish a benchmark for long-term monitoring and management.

(3). Defining water quality and inter-species interactions at a

regional level, and identifying priority areas for restoration, at least in part, based on these interactions.

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MUSSEL *MYTILUS EDULIS* (L.) FILTERING OF THE BALTIC SEA OUTFLOW THROUGH THE ÖRESUND—AN EXAMPLE OF A NATURAL, LARGE-SCALE ECOSYSTEM RESTORATION

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ABSTRACT Investigations were undertaken to quantify the filtering capacity of mussels at the sill in Öresund, one of the straits connecting the Baltic Sea with the Kattegat. The investigations included observations of hydrography and currents and measurements of nutrients, chlorophyll-*a*, and phytoplankton. Tracing the water over the vast mussel banks at the sill, we found that about 75% of the phytoplankton biomass was removed from the water. The clearance rate, defined as the water volume cleared of organic matter per unit time and unit ash-free dry-weight of soft tissues of the mussels (AFDW), was estimated at 7 L/h/g. Based on the mussels present at the sill (Madsen and Höjgård Petersen 1996), we determined that the mussels could clear the outflow from the Baltic Sea through Öresund almost completely of phytoplankton biomass. We also found that the plankton biomass recovered after the passage of the sill.

KEY WORDS: Mussel filtering, clearance rate, ecosystem restoration, Öresund

INTRODUCTION

Benthic suspension-feeders like mussels (*Mytilus edulis*) can have a dominant influence on the flux of nutrients. This has been found in various environments of Scandinavia: the Oslo Fjord in Skagerrak (Kirkerud and Bjerkeng 1994), the Roskilde Fjord in the Kattegat (Möhlenberg 1995), and the Askö archipelago in the Baltic Sea (Kautsky and Wallentinus 1980). These examples represent regions with salinities ranging from more than 30 to 5 psu. Cloern (1982) suggested that bivalves controlled the phytoplankton biomass and prevented plankton blooms in the shallow estuary of South San Francisco Bay, which receives effluent from 20 municipal sewage treatment plants. All these examples are from areas experiencing some level of eutrophication. Filtering by bivalves decreases the negative effects of eutrophication in three ways: (1) by improving light conditions, (2) by decreasing the flux of organic matter to deeper layers with limited water exchange, and (3) by leveling primary production. The first two ways are direct effects of filtering. In the third case, filtering decreases primary production during plankton blooms and remineralized nutrients are supplied to the water over prolonged periods.

In situ measurements of nutrient uptake and remineralization by mussel beds have been made in tunnel and flume experiments by Dame and Dankers (1988), Prins and Smaal (1990), and Asmus and Asmus (1991). The common mussel was the dominant species in these experiments, contributing to approximately 90% of the total macrobenthic biomass. In the study by Asmus and Asmus (1991), the uptake of chlorophyll-*a* (Chl-*a*) and the release of inorganic nutrients were determined in water that passed through a 20-m long tunnel enclosing a mussel bed. In their experiments phytoplankton biomass was reduced by 37±20%.

In the present study, Öresund, one of the three straits connecting the Baltic Sea with the Kattegat and the North Sea (see Fig. 1), was chosen as the site to measure the influence of mussels on inorganic nutrients and phytoplankton fluxes. The sill is situated between København, Denmark, and Malmö, Sweden (Fig. 1), and is covered with a large mussel bank. Here, grazing by other consumers of seston could be neglected in comparison with the filtering by mussels. In this area it was possible to trace the water several kilometers over the mussel bank, corresponding to several hours, which is a much larger scale than in the tunnel experiments cited. The biomass of the phytoplankton community during and after the water passed over the mussel banks could also be studied.

The strategy for the investigation was to trace the water over the sill. Observations of hydrography and currents were performed together with water sampling for the determination of nutrients, Chl-*a*, and phytoplankton.

In the following sections the topography and the mussel population in the sill area, the large-scale hydrographic conditions, and methods of data collections are discussed. A simple model that quantifies filtering by the mussels is presented, and conclusions about the effects of filtering in general and on the out-flowing Baltic Sea surface water in particular are drawn. The detailed investigation of the plankton community is presented in a separate study, Norén et al. (In press).

THE SILL AREA: TOPOGRAPHY AND MUSSEL POPULATION

The sill in Öresund, the Drodgen sill, has a minimum cross-sectional area of about $60 \cdot 10^3$ m^2. The minimum width is 14 km and the depth ranges from 3 to 10 m. The horizontal area of the sill is about 170 km^2 and the length of the sill in the main current direction is about 14 km (Fig. 2). The ground of the sill can be characterized as a transport bottom. It consists of limestone, hard clay, boulders, stones, and coarse sand. This substrate is preferred by the red algae (*Furcellaria lumbricalis*, J. V. Lamouroux) and when mussel larva first settle, often it is on the seaweed. When the mussels are larger they move to the hard bottom substrate and as a result, vast areas of the sill are almost totally covered by mussels.

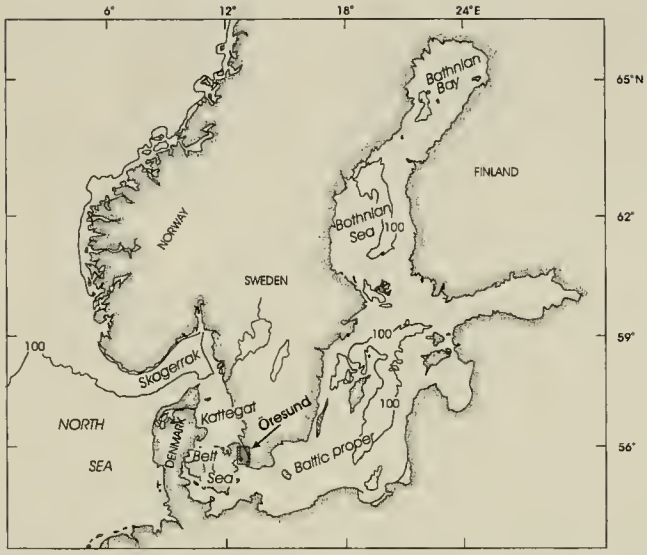

Figure 1. The location of Öresund, between the Baltic Sea and the North Sea (100 m depth contour indicated).

Due to low and highly variable salinity (see Hydrography), mussels grow slowly and seldom reach a size of more than 40 mm. Most mussels are in the size range 5–13 mm (Madsen and Höjgård Petersen 1996). Due to the low salinity, starfishes are absent in the area and crabs are few; otherwise, these are usually the most common mussel predators. However, a third predator, the eider duck, is common in the Öresund sill area, with about 450,000 bird days per year at Saltholm (Noern and Christensen 1997).

The investigations by others of the possible impact on the marine environment caused by the construction of a link across the Öresund included video and echo sounding surveys and samplings of the mussel population in the sill area. During the period 1990 to 1996, divers sampled 800 stations to determine coverage, biomass, and size distribution of the mussels. Mussels occur throughout the area (Fig. 2), with the densest population in the Flintrännan between Saltholm and the Danish coast. There, the coverage of mussels is 70–100% by area. In 1996, the areas with more than 40% coverage of mussels were estimated to be 128 km^2 (Fig. 2) and the average biomass, in wet weight of soft parts and shell, was 7.08 kg/m^2 (Madsen and Höjgård Petersen 1996). Wet weight (WW) of mussels with shell is converted to ash-free dry-weight of soft tissues (AFDW) using a conversion factor of 0.03 (the tissue content in these mussels is extremely low due to poor growing conditions). The mussels in the sill area were estimated at about 900,000 tons WW, which corresponds to 27,000 tons AFDW or 200 g AFDW/m^2.

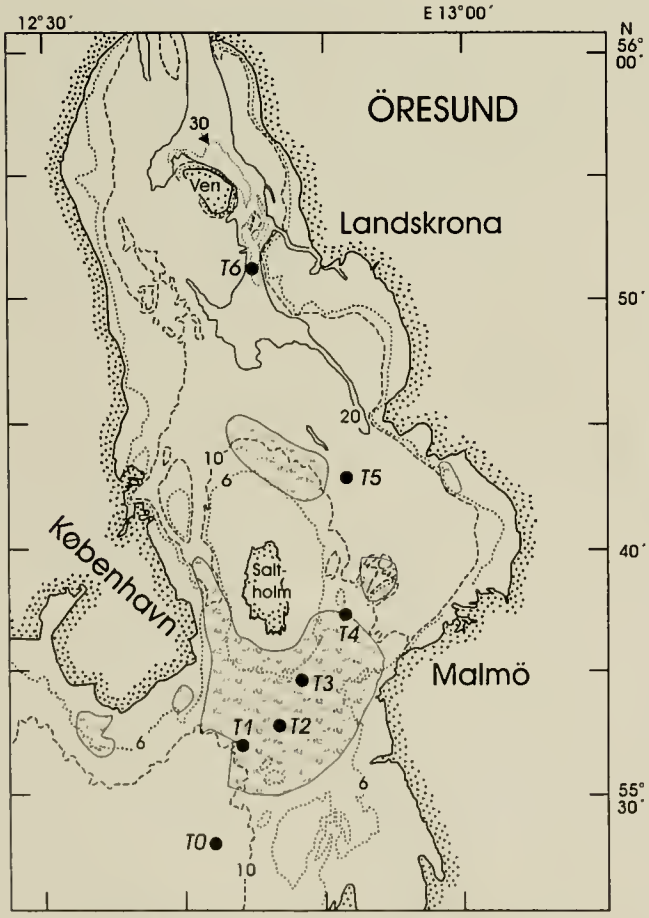

Figure 2. Topography of the Öresund with depth contours for 6, 10, 20, and 30 m. Mussel banks in the vicinity of the sill (shaded areas) and sampling stations are indicated.

HYDROGRAPHY

The large-scale hydrographic conditions in the Baltic and the North seas are discussed in Rodhe (1998). The connection between these seas is through three parallel straits, one of which is Öresund. The flow over the sill in Öresund is determined mainly by the alternating difference in water level between the southern part of the Baltic Sea and the Kattegat, on the North Sea side of Öresund. The difference in salinity between the surface waters of the southern part of the Baltic Sea and the Kattegat, about 10 psu, is of minor importance to the flow rate due to the shallowness of the sill. On the Baltic Sea side of the sill a halocline separates the Baltic Sea's low salinity surface water from the more saline deep water; the halocline is situated far below the depth of the sill. In Öresund to the north of the sill, the water is stratified and the halocline is at a depth of 10 to 20 m. Below that, the salinity exceeds 30 psu. The salinity in the sill area varies between 8 and 24 psu. However, intense vertical mixing at the sill keeps the water vertically homogeneous, except in cases of very weak current.

The average annual outflow from the Baltic Sea through Öresund is $30.3 \cdot 10^{10} m^3$ (9600 m^3/s), with an average salinity of 9.5 psu, which is close to the average salinity of the Baltic Sea surface water inside the sill. The average annual inflow to the Baltic Sea is $17.4 \cdot 10^{10} m^3$ (5500 m^3/s), with an average salinity of 13.3 psu (Svensson et al. 1994, Mattsson 1996).

A long time-series (1931–1976) of current registrations at the Drogden light vessel showed two dominating current directions: 50° and 230°, which represent inflow and outflow, respectively. During this 45-y period, flow was northward 52% of the time, with an average speed of 27 cm/s, and southward 32% of the time, with an average speed of 40 cm/s (the direction of current velocities < 5 cm/s was not noted). The highest observed speed during northward flow was 175 cm/s, with an estimated transport of 105,000 m^3/s. The maximum velocity during southward flow was 225 cm/s, which corresponds to a discharge of 125,000 m^3/s. The average duration of northward flow and southward flow was 1.5 days and 1.2 days, respectively. The longest observed period of northward flow was 23.3 days and of southward flow 11.5 days. The salinity variations from these alternating flow directions affect the metabolism of the mussels, resulting in decreased growth.

OBSERVATIONS AND METHODS

We were interested in two processes: (1) filtering by mussels when water passes over the mussel bank, and (2) the development of the phytoplankton community after the water's passage over the sill. Our main observations were made during a persistent outflow from the Baltic Sea. The water leaving the sill area continues as surface water over the deep and stratified part of Öresund to the north of the sill, and the phytoplankton community develops in the surface layer without contact with the bottom. On the other hand, during flow toward the Baltic Sea, water subducts below the low-salinity surface layer after passing over the sill, due to its higher salinity.

Our main investigation was carried out onboard the R/V *Skagerak*, of Göteborg University, during 2 days in May 1997. In addition, the Swedish Meteorological and Hydrological Institute has supplemented the investigation by sampling some of the stations during different seasons in 1997 and 1998 (sampling was performed from onboard the R/V *Argos*).

The observations in May 1997 were carried out along a track passing over the sill (Fig. 2). The stations were chosen so that stations T1 to T4 were situated on the shallow part of the sill (depth about 8 m), where we expected the water to be well mixed vertically. These stations were all well within the mussel-bank region. Station T0 was situated a short distance to the south, and T5 a short distance to the north of this region. Station T6 was situated in the deep and stratified part of Öresund. The track essentially followed the direction of the current. However, the time to make the observations along the track was about half of the time needed for the water to be advected along the track, indicating that we did not follow exactly the same water mass.

During the 2 days of sampling in May 1997, three profiles were made. On May13 the stations T1 to T4 were sampled twice within 5.5 h. On May 14 stations T0 to T6 were sampled once within 7 h. The R/V *Argos* sampled the stations within about 3 h.

Temperature and salinity were measured with a CTD (Niel Brown MK 5). Nutrients (total N, total P) were analyzed with an autoanalyzer (Alpkem/RFA2) according to methods recommended in The New Baltic Manual (Carlberg 1972). Water samples for Chl-*a* analysis were taken with Niskin bottles at one or two depths (see Table 1a and b). The 100-ml samples were filtered through GF/F filters, and the filtrate was extracted in 90% acetone for12 h at 4°C. Chl-*a* determination was made fluorimetrically according to standard methods. Currents were measured at 0.5, 1, 2, 3, 4, 6, 8, and 9 m above the seabed with pendulum current meters (Cederlöf et al. 1996) at every station except T6, where no current observation was made. In addition, current data were provided from fixed current meters at Flinten SV (close to station T3 in Fig. 2), 5.1 and 8.1 m above the seabed.

RESULTS

As expected, the water temperature and salinity was almost vertically homogeneous in the shallow stations of the sill area. Also, the horizontal variations were small. Exceptions were April 20, 1998, June 23, 1998, and August 27, 1998, when both vertical and horizontal gradients were found, showing that the observations were made in different water masses. However, the current was unidirected in the vertical in all observations. The observations are presented in Table 1.

General conclusions from the observations are:

(1). The changes in total phosphorus and nitrogen concentrations were relatively small, and showed no systematic decrease or increase following the water's passage over the sill. This was expected because the majority of the nutrients were dissolved, and thus would not be removed from the water as a result of the mussels' filtering. Also, nutrient inputs due to excretion from the mussels were too small to be of any appreciable influence on total nitrogen and phosphorus, see below.

(2). In some cases, large vertical and horizontal changes in ammonium concentrations were found. This indicates strong local sources or sinks. Otherwise, it would have been homogeneous, as were temperature and salinity. In a majority of the observations the concentration of ammonium increased, following the passage of the water over the sill, both in the northerly and the southerly direction. This increase in ammonium could be due to excretion from the mussels. Note that in two of these samples there were differences in salinity, indicating that the observations were made in different water masses.

(3). Large, systematic changes in the Chl-*a* concentration were observed on all occasions, except in the one case with weak currents and a large horizontal salinity gradient. A majority of the observations showed a somewhat lower value at the bottom. Figure 3a shows observations of NH_4-N versus Chl-*a*, from which it is obvious that there is a negative correlation between the two parameters. This supports the idea that the mussels remove phytoplankton from the water and, at the same time, excrete ammonium. The fact that neither NH_4-N nor Chl-*a* shows systematic variation with salinity (Fig. 3b and c) indicates that the inverse correlation between NH_4-N and Chl-*a* is related to a local source-sink and is not an indication of different water masses that are advected over the sill.

Figure 4 shows the change in chlorophyll concentration along the track in May 1997 (see also Table 1a). The effect of the mussels' filtering is striking. An approximately 75% decrease in the Chl-*a* concentration was observed when the water passed over the mussel bank, changing from about 1.1 mg/m^3 at station T0 to about 0.3 mg/m^3 at station T4. After the water passed over the mussel bank the concentration again increased, to about 1.4 mg/m^3

TABLE 1a.

Station	Date YYMMDD	Depth m	T °C	S psu	Tot P µmol/L	Tot N µmol/L	Chl-*a* mg/m^3	Dir (degree) Velocity (cm/s)
T1	970513	3	7.4	8.4	0.5	19	0.87	50/28
T1	970513	9	7.4	8.4	0.4	18	0.63	
T2	970513	1	7.6	8.4	0.4	15	0.55	50/28
T2	970513	7	7.6	8.4	0.4	15	0.47	
T3	970513	1	7.6	8.4	0.4	17	0.30	50/45
T3	970513	6	7.6	8.4	0.4	17	0.47	
T4	970513	1	7.9	8.4	0.4	15	0.30	50/31
T4	970513	1	7.9	8.4	0.5	15	0.22	
T1	970513	4	7.5	8.4	0.4	16	1.20	50/45
T3	970513	4	7.7	8.4	0.4	16	0.47	50/35
T4	970513	4	7.6	8.3	0.4	15	0.28	50/32
T0	970514	1	7.9	8.4	0.5	18	1.20	50/28
T0	970514	6	7.9	8.4	0.6	17	1.00	
T1	970514	1	7.8	8.3	0.5	15	0.82	50/42
T1	970514	6	7.8	8.3	0.5	16	0.63	
T2	970514	1	7.9	8.3	0.5	15	0.65	50/40
T2	970514	6	7.9	8.3	0.5	15	0.53	
T3	970514	1	7.9	8.3	0.5	15	0.48	50/60
T3	970514	6	7.9	8.3	0.4	15	0.48	
T4	970514	1	7.9	8.3	0.5	15	0.33	50/51
T4	970514	6	7.9	8.3	0.4	15	0.31	
T5	970514	1	8.3	8.4	0.4	15	0.84	50/23
T6	970514	3	8.4	8.6	0.5	15	1.40	

Temperature (T), salinity (S), total phosphorus (Tot-P), total nitrogen (Tot-N), chlorophyll-*a* (Chl-*a*), and vertical average of the current (Direction/Velocity) as observed during May 1997 (R/V Skagerak).

at station T6. (In the calculations later changes in Chl-*a* concentration were assumed to reflect changes in the phytoplankton biomass. This is a reasonable assumption when we track a water mass over several hours in the middle of the day.)

Was the observed increase in Chl-*a* the indicative of recovery of the phytoplankton community within the same water mass, and with the available nutrients? We do not know for sure, but the indications are strong. A rough estimate of the time to advect water from station T4 to T6, based on the current measurements, is 2 days. Two doublings of biomass during that time is quite realistic. Also, based on temperature, salinity, total phosphorus, and nitrogen measurements from the 2 days of sampling in May 1997 (see Table 1a), we conclude that the observations were made within the same water mass and that mixing with the underlying water was negligible. Our conclusion is that the observations at stations T4 to T6, shown in Fig. 4, indeed show the development of the phytoplankton community in a water mass exposed to the effective filtering by mussels and, possibly, supplied with ammonium.

The detailed current observations from May 1997 were used to calculate the bottom stress, formulated as friction velocity (u_*), and the drag coefficient (c_d), relating the velocity to the bottom stress. The definitions of these quantities are

$$(u_*)^2 = \tau_0/\rho$$

where τ_0 is the bottom stress and ρ is the water density, and

$$c_d = (u*/u_m)^2$$

where u_m is the velocity, averaged in the vertical direction. The friction velocity was estimated by fitting the current observations to a logarithmic profile at each station. Results of the calculations are shown in Table 2 together with the depth-averaged velocity at each station. The small variations in the direction of the current were not considered in the calculations. Although the calculations were made using single observations, we believe that estimated friction velocities are the correct order of magnitude. The comparatively large drag coefficients (the average is $7.0 * 10^{-3}$) seem to be realistic since the ground is very rough (see the section The Sill Area). In addition, the mussel beds at the bottom increase the bottom friction by increasing the bottom roughness. Further, the mussels' pumping activity increases the bottom friction (Westerberg unpubl.). The current observations and the friction velocities are used in the calculations that follow.

QUANTIFICATION OF FILTERING USING AN INTERPRETATION MODEL

An interpretation model was formulated to apply values to the filtering by the mussels. The model description follows: as the water flows over the mussel bed, turbulence keeps the biomass evenly distributed vertically. The mussels filter a fraction of the water column per unit length in the flow direction. The size of this fraction depends on the velocity, the depth of the flow, and filtering by the mussels. Because observations of changes within a water mass are made over a few hours, Chl-*a* concentration was treated as being proportional to the biomass and changes in Chl-*a* were used to calculate the amount of biomass filtered. The observations with high horizontal resolution and simultaneous current measurements (those in 1997) were used. To make the model as simple as possible, we assumed the flow properties were horizon-

TABLE 1b.

Station	Date YYMMDD	Depth m	T °C	S psu	Tot P µmol/L	NH_2-N µmol/L	Tot N µmol/L	Chl-*a* mg/m^3	Dir (degree)/ Velocity (cm/sec)
T0	971215	2	5.3	7.9	0.36	0.2	22.6	0.9	50/75
T0	971215	8	5.4	7.9	0.36	0.27	18.9	0.9	50/75
T1	971215	2	5.4	8.2	0.59	0.27	22.7	1.1	50/75
T1	971215	7	5.4	8.4	0.62	0.31	23.9	1.0	50/65
T2	971215	2	5.4	8.2	0.60	0.28	21.9	1.0	50/75
T2	971215	8	5.4	8.2	0.60	0.63	21.2	1.0	50/65
T3	971215	2	5.4	8.5	0.62	0.37	33.3	1.1	50/75
T3	971215	7	5.4	8.5	0.61	0.28	21.5	0.9	50/65
T4	971215	2	5.3	8.4	0.57	0.75	23.3	0.8	50/68
T4	971215	7	5.3	8.5	0.61	0.73	20.7	0.6	50/60
T5	971215	2	5.1	8.6	0.65	0.62	20.4	0.8	
T0	980319	0	3.6	9.3	0.86	1.05	25.4	0.5	50/30
T0	980319	8	3.6	9.3	0.65	1.00	25.4	0.4	40/30
T4	980319	0	3.6	9.8	0.36	0.73	21.3	0.6	40/30
T4	980319	7	3.6	9.8	0.37	0.89	21.8	0.7	40/30
T0	980420	0	5.6	7.5	0.56	0.14	17.8	1.2	230/5
T0	980420	7	6.5	10.3	0.50	0.13	18.9	1.2	230/5
T4	980420	0	6.4	11.4	0.57	0.13	19.6	1.5	230/10
T4	980420	7	5.5	22.4	0.93	0.59	19.2	1.9	230/10
T0	980603	2	12.1	7.4	0.10	0.14	18.4	1.5	50/30
T0	980603	7	12.1	7.5	0.10	0.13	18.7	1.5	50/30
T4	980603	2	13.3	8.1	0.02	0.15	21.0	1.3	40/35
T4	980603	7	13.2	8.2	0.15	0.12	18.6	1.3	40/35
T0	980623	2	13.7	7.5	0.48	0.10	21.1	2.5	230/10
T0	980623	7	13.6	7.8	0.47	0.22	23.6	1.9	230/10
T4	980623	2	14.3	8.8	0.53	0.14	22.2	2.0	230/10
T4	980623	7	14.8	10.1	0.48	0.20	19.3	1.3	230/10
T0	980721	2	14.4	7.8	0.54	0.12	19.8	1.8	50/50
T0	980721	7	14.4	7.8	0.59	0.12	21.0	1.8	50/50
T4	980721	2	15.0	9.2	0.62	0.69	19.3	0.8	20/40
T4	908721	7	15.0	9.3	0.61	0.78	19.2	0.6	20/40
T0	980823	2	15.6	15.9	0.25	0.67	16.8	0.5	220/85
T0	980823	7	15.6	16.0	0.26	0.68	17.3	0.4	220/65
T4	980823	2	15.7	17.2	0.18	0.21	15.7	1.7	220/85
T4	980823	7	15.7	17.2	0.20	0.26	17.2	1.4	220/85
T0	980827	2	15.1	12.3	0.19	0.10	17.9	3.7	40/40
T0	980827	7	15.3	13.7	0.21	0.08	17.3	2.9	40/35
T4	980827	2	15.0	16.6	0.27	1.16	17.5	1.2	40/45
T4	980827	7	15.0	16.6	0.28	1.21	17.6	1.1	40/35
T0	980922	2	14.6	7.6	0.02	0.12	20.6	2.8	40/31
T0	980922	7	14.6	7.6	0.04	0.09	21.2	6.8	40/31
T4	980822	2	14.4	7.9	0.30	1.02	19.9	0.5	40/41
T4	980922	7	14.4	7.9	0.28	1.22	20.3	0.5	40/41

Temperature (T), salinity (S), total phosphorus (Tot-P), ammonium (NH_4-N), total nitrogen (Tot-N), chlorophyll-*a* (Chl-*a*), and vertical average of the current (Direction/Velocity). Five observations from December 1997 to August 1998 (R/V Argos).

tally homogeneous over the sill. This assumption is based on the relative homogeneity of the depth distribution and the bottom structure. However, the degree to which this assumption is fulfilled does not change the result in a qualitative way, it only effects its quantitative accuracy. Another assumption is that the local change in biomass with time is small compared with the change over time following water flow over the sill. This assumption is critical for the result, and the similarity among the observations from the three crossings of the sill indicates it is well supported (see Fig. 4).

Before proceeding with the calculations, the question must be answered whether the vertical mixing is vigorous enough, that is, if all the water between the observation sites potentially can be filtered by the mussels. If not, then the mussels' filtering will only affect water in the vicinity of the bottom. Fulfilling the flow criterion requires that the vertical turnover time be considerably shorter than the time for the water to be advected between the sites. In the present situation the vertical turnover is determined by the turbulence generated by the bottom friction. Thus, the friction velocity can be used as an estimate of the turbulent velocity. The condition to be fulfilled is:

$$h/u' < l/U \qquad (1)$$

h, u′, l, and, U are the depth, the turbulent velocity scale, the advective length, and the advective velocity, respectively.

Using information from Table 2 and Fig. 2 to estimate the magnitude of the terms in Eq. (1), h = 10 m, u′ = 0.02 m/s, U = 0.5 m/s, and l = 3000 m. Using these values, the vertical turnover

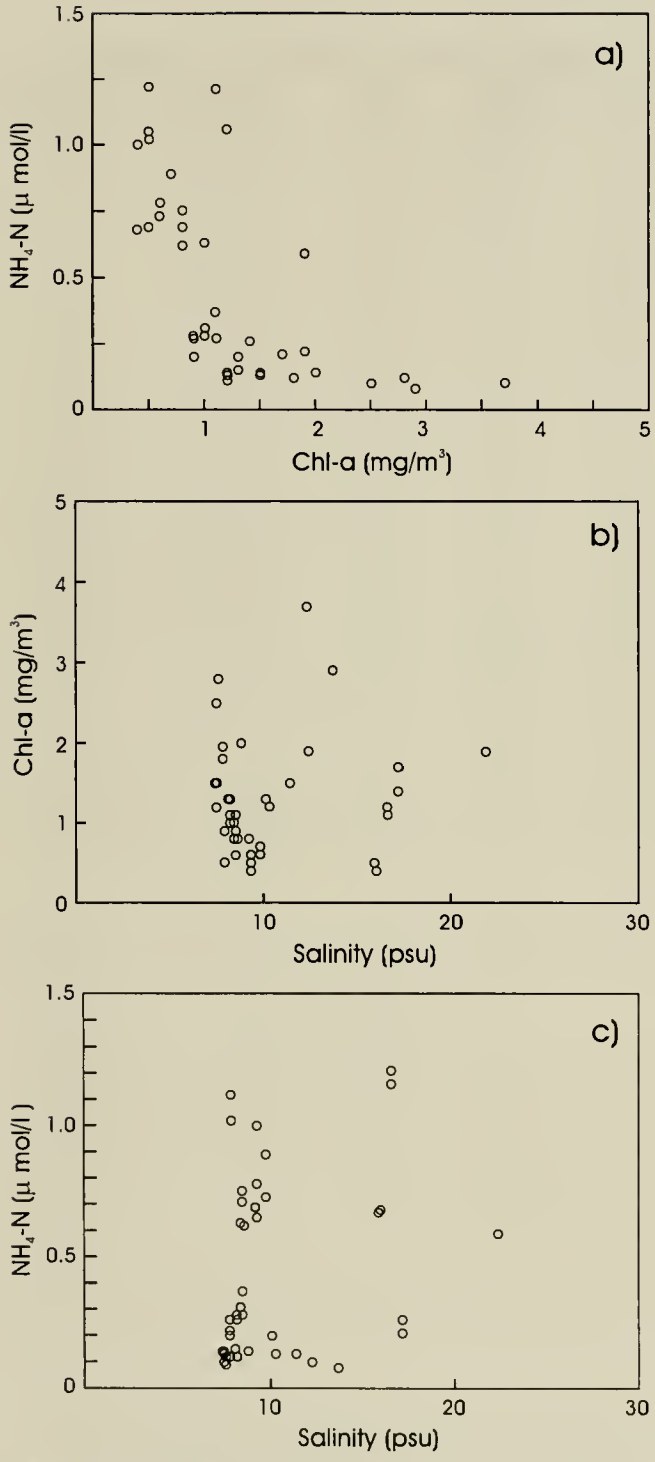

Figure 3. (A) Observations of NH_4-N versus Chl-*a*., (B) Chl-*a* versus salinity, and (C) NH_4-N versus salinity.

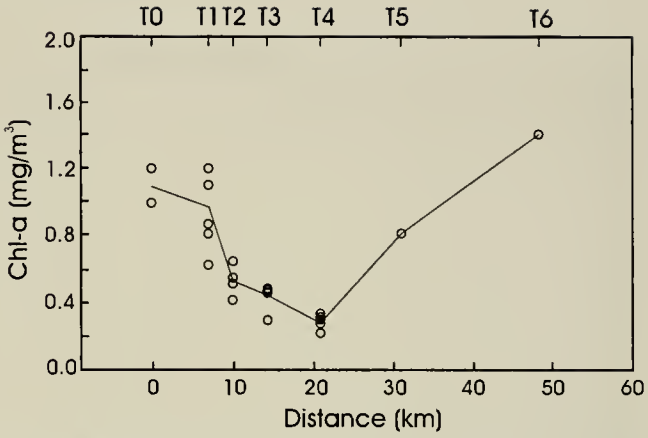

Figure 4. Observations of Chl-*a* concentration along the transect crossing the sill in Öresund. Data from the observations in May 1997 (Table 1a). The line connects the ensemble average at each station. The sampling stations are shown in Figure 2.

time (left side of Eq. 1) is 500 s and the advection time is 6000 s. We conclude that the condition (Eq. 1) is fulfilled.

We also assumed that the water filtered by the mussels is "immediately" mixed in the water column. This is supported if the flow of water through the mussels at the bottom, converted to a vertical velocity, is much smaller than the turbulent velocity. This will be confirmed a posteriori. The model set up is illustrated in Figure 5. We formulated a conservation equation for chlorophyll in a one unit wide channel, where the horizontal transport of chlorophyll units by the current is determined by the vertical average of the velocity (U) multiplied by the depth (h) and by the vertical average of the chlorophyll concentration (C). (This is reasonable because the vertical gradient of the concentration was small.) The loss of chlorophyll, per unit area, due to the filtering by mussels is formulated as an area-average velocity (w_m), representing the filtering by mussels multiplied by the local chlorophyll concentration. The change over a short distance, and per unit length in the x-direction, of the horizontal transport can be formulated:

$$d/dx(UhC) = -w_m C \tag{2}$$

To solve the equation, U and h will be treated as constants. (To do this we assumed that the flow is horizontally homogeneous.) The solution to Eq. (2) then is

$$C/C_0 = \exp[-(w_m/Uh)x] \tag{3}$$

where C_0 is the concentration at $x = 0$.

Solving for w_m:

$$w_m = -\ln(C/C_0) \cdot Uh/x \tag{4}$$

Equation (4) is used between successive observational sites to estimate w_m. The average value of Uh is used at each pair of sites. The results are presented in Table 3. The scatter in the calculated w_m is in the authors' opinion astonishingly small. The estimated mean is $0.4 \cdot 10^{-3}$ m/s. Note that this is about two orders of magnitude smaller than the turbulent velocity. We also see that there seems to be no systematic variation of w_m, neither with the average velocity nor with the velocity at the bottom. This fact supports the calculations.

The clearance rate (CR) is defined as the water volume cleared of organic matter per unit time and unit AFDW of mussels. We can use the velocity, w_m, to estimate CR. The average mussel density at the sill is about 200 g/m^2, measured as AFDW. Thus, the estimated CR is about $2 \cdot 10^{-6}$ m^3/s/g AFDW, which is approximately equal to 7 L/h/g AFDW. This compares well with values given by Jörgensen (1990) for small mussels. A rough estimate of the clearance rate by the entire mussels population on the sill, 27,000 tons

TABLE 2.

Flow properties calculated using the detailed curent measurements from May 1997.

Date YYMMDD	Station	Depth m	U cm/s	u_* cm/s	c_d $*10^3$
970513/1	T0	12	no	no	no
	T1	10	27.6	2.3	6.9
	T2	9.5	26.9	2.1	6.1
	T3	9	44.9	1.6	1.3
	T4	9	31.0	2.6	7.0
970513/2	T0	12	no	no	no
	T1	10	33.5	3.0	8
	T2	9.5	no	no	no
	T3	9	37.1	4.1	12.2
	T4	9	25.5	2.0	6.2
970514	T0	12	24.6	2.3	8.7
	T1	10	39.4	2.6	4.4
	T2	9.5	37.7	3.4	8.1
	T3	9	55.3	4.2	5.8
	T4	9	46.2	4.4	9.1

U is the depth-averaged velocity, u_* is the friction velocity, and c_d is the drag coefficient (no = not observed).

AFDW, then becomes $5 \cdot 10^4$ m^3/s. This figure is more than two times the average flow rate over the sill during outflow from the Baltic Sea. Even though this is a rough estimate, we conclude that the mussels at the sill have the capacity to filter the outflowing water from the Baltic Sea.

DISCUSSION OF LOCAL AND LARGE-SCALE EFFECTS OF FILTERING BY MUSSELS

In our study we have assigned numerical values to the filtering by the mussels in Öresund. The local effect of the mussels is obvious to everyone who visits the sill area: the water is extremely clear. Therefore, one can find flourishing fields of eelgrass (*Zostera marina* L.) down to 7 m deep and *Laminaria saccharina* (L.) down to 14 m in Öresund. A large supply of nutrients, remineralized from the mussels during summer, when a shortage of nutrients normally occurs, could be an additional contributor to the good growth of SAV.

The input of ammonium from mussel banks due to the metabolism of the mussels is considerable. The amount fluctuates due to changes in temperature, salinity, nutrient supply, and the physiological status of the mussels (Smaal et al. 1997). Our observations also indicated a release of ammonium from the mussel banks. In the observations from December 1997, the NH_4-N concentration increased from about 0.3 μmol/L to about 0.7 μmol/L after the water passed over the mussel bank. The water transport through Öresund was about 36,000 m^3/s (a rough estimate based on current observations). Assuming that the observed increase in the ammonium concentration is representative, we estimated the total release of NH_4-N to be 14 mol/s, or 0.7 ton/h, from the mussel banks at the sill. During northward-directed flow such a release constitutes an appreciable, accessible supply of nutrients to the central part of Öresund (see Fig. 2). The total production in Öresund is estimated at 91 g C /m^2/year (Mattsson 1993). For assimilation of carbon to take place, nitrogen is needed; in this case about 1.3 ton/h, on the average. Using the Redfield ratio to relate C to N, the ammonium released from the mussel banks can be expected to have a large

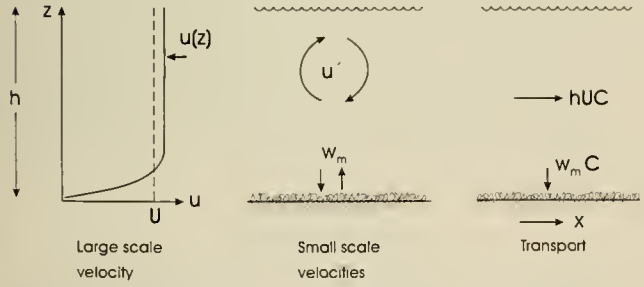

Figure 5. Conceptual model used to calculate filtering by mussels. Water flows with velocity u(z) over the mussel bed. The total depth is h. Bottom friction induces turbulence, characterized by a velocity, u'. The filtering by mussels induces a vertical velocity, w_m, at the bottom. The biomass, measured as Chl-*a* concentration, C, is advected with mean current, U, in the x-direction. The filtering by mussels at the bottom acts as a sink for biomass. This sink is expressed as a vertical velocity multiplied by the chlorophyll concentration.

TABLE 3.

Calculations of the vertical velocity (w_m) related to the filtering by mussels between different station pairs.

Date YYMMDD	Station Pair	W_m mm/s	U cm/s	$u_{0.5}$ cm/s
970513/1	T1–T2	0.7	28	17
970513/1	T2–T3	0.2	36	26
970513/1	T3–T4	0.3	38	26
970513/2	T1–T3	0.5	35	16
970513/2	T3–T4	0.3	31	14
970514	T1–T2	0.3	39	19
970514	T2–T3	0.3	46	20
970514	T3–T4	0.4	51	31
971215	T1–T4	0.3	69	no
Ensemble average		0.4	41	21
Std. deviation		0.2		

U is the depth mean velocity, averaged between the stations, and $u_{0.5}$ is the velocity 0.5 m above the bottom (no = no observation).

effect on primary production, in a qualitative as well as in a quantitative way.

The concentration of inorganic nutrients is higher in Öresund compared with what is found in the adjacent seas (Mattson 1993). Despite that, the measured pelagic primary production is lower in the vicinity of the sill than in the Kattegat, in the northern part of the Öresund, and in the southern part of the Baltic Sea. Edler (1977) suggested that the low production is a result of the rapid salinity changes in the area. The reduction of the chlorophyll content in the water, by the mussels at the sill, might be an alternative cause of low productivity in the sill area. Tracing the water close to the sill, we found the beginning of a bloom: nutrient-rich water but few phytoplankton.

Increased nutrient concentration in the Kattegat, in the Belt Sea, and in the Baltic proper has led to a doubling of summer primary production in these areas since the mid 1960s (Shultz et al. 1990). Vast bottom areas in the southern part of Kattegat have suffered from temporary anoxia since the beginning of the 1980s, probably as a result of an increased flux of organic matter from the photic zone to the deeper layers (Anderson and Rydberg 1988). The oxygen demand has also increased in the deep water of the Baltic Proper. The opposite is true for the central part of Öresund, where observations indicate a decrease in oxygen demand in the deep water. Mattsson (1993) found a 30% decrease in oxygen consumption between 1967 and 1986 in the Landskrona Basin water, which is an isolated trough situated close to station T6 (see Fig. 2). The maximum depth is 50 m and exchange with the deep water of the Kattegat to the north is hampered by a 25-m deep sill. Mattsson (1993) found this decrease surprising, considering the increased nutrient concentration in the surface layer. His conclusion was that the decrease in the oxygen consumption in the basin water reflected a decreased net production in the surface layer.

However, there is another possible reason for the decreased flux of organic matter to the deep water in Öresund. Kautsky and Wallentinus (1980) suggested that mussel populations in regions with few predators expand to the carrying capacity of the area. As stated earlier the Öresund sill area is such a region. Consequently, we assume that the mussel banks at the sill have grown during the last decades due to eutrophication, and that the mussels' capacity for filtering is determined by the peak in the supply of organic matter. In fact, according to local fishermen the banks have grown during the period, but there is little scientific documentation. The supply of organic matter to the mussels varies with the water flow and the season of the year. Consequently, during most times of the year, when the nutrient supply is moderate, the starving mussels can filter the passing water more efficiently than before. The total effect would probably be a net decrease in the amount of organic matter that could escape from being filtered by the mussels. The effect on the oxygen conditions in the Landskrona Basin would be twofold: The filtering of seston from water flowing from the Baltic Sea to the Öresund will decrease flux to the deep water. We can also expect that "new" production, in the water filtered by the mussels, implies a shift of the phytoplankton community toward smaller species. Smaller plankton settles slower and decomposes faster. This means that a smaller portion of the organic matter produced in the surface layer settles before the water leaves the Öresund.

There is reason to believe that the mussel banks in Öresund accumulate large amounts of nutrients when there is excess of food for the mussels during the spring phytoplankton blooms. Also, they contribute to the nutrient accumulation with a net release of nutrients during the summer (Asmus and Asmus 1991). The implied leveling effect on the nutrient fluxes should be important for other species in the Öresund ecosystem.

The present study is a preliminary study for a project investigating the potential improvements in water quality from mussel cultivation in eutrophicated areas of the Swedish west coast (e.g., the Orust-Tjörn fjord system to the north of Göteborg). The deep basins in these fjords often suffer from low oxygen concentration in late summer and autumn due to restricted water exchange and a large supply of organic matter from the surface layers. The populations of filter feeders in these fjords have increased during the last decades due to eutrophication, but they have not reached the carrying capacity. There has been a small decrease in the flux of organic matter to the deeper layers in the inner parts of the fjord system (Kajrup 1996), but it is not possible to isolate the effect of increased water purification from that of an increased population of filter feeders. Future work will focus on the potential for decreasing BOD in the deep basin water by shifting part of the flow of organic matter to mussel culture. Long-line culture of mussels has been practiced on the Swedish west coast since 1970 with positive results for settling of larva and for growth in many locations. In the future we hope the results of the investigation from the sill in Öresund will help us site mussel farms in areas where the filtering activity of mussels can counteract the negative effects of eutrophication.

CONCLUSIONS

We have found that the mussels at the sill in Öresund, through their filtering, are capable of clearing phytoplankton almost completely from the passing water; and the remineralization by the mussels can be expected to have a first-order effect on the ecosystem in Öresund, in a qualitative as well as in a quantitative way.

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TEMPORAL AND SPATIAL DISTRIBUTION OF ENTEROCOCCUS IN SEDIMENT, SHELLFISH TISSUE, AND WATER IN A NEW ZEALAND HARBOUR

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ABSTRACT Enterococci, a group of faecal bacteria commonly found in stormwater discharges, were used to trace the spatial and temporal impact of waste streams from an outfall in the Whangateau Harbour, northeastern New Zealand. A seasonal trend in levels of enterococci in two infaunal bivalves, *Austrovenus stutchburyi* (Gray in Wood 1828) and *Macomona liliana* (Iredale 1915) was detected, with maximum contamination correlating with high winter rainfall. Rainfall events were also shown to affect stormwater and harbor water significantly. Median enterococci levels in *A. stutchburyi* were higher at the putative impact site compared to the reference site, and were higher than *M. liliana* at both sites. Bacterial levels in surficial sediment and *A. stutchburyi* tissue declined with distance from the stormwater outfall and the stormwater channel. Enterococci were effective for determining the spatial and temporal patterns of stormwater discharge in this harbor, and may have general applicability as an indicator of such discharges.

KEY WORDS: Stormwater, enterococci, *Austrovenus stutchburyi*, cockle, *Macomona liliana*

INTRODUCTION

There is a paucity of literature concerning temporal and small-scale spatial effects of stormwater discharges on the bacteriological quality of the marine environment adjacent to small coastal settlements in New Zealand. Typically, these settlements comprise dwellings along harbor or estuary fringes, with stormwater flows discharged directly onto the intertidal zone, which is inhabited by many edible shellfish species. Long-term monitoring of bacterial contamination is needed to establish average or background levels, which in turn assist in the interpretation of short-term event-driven episodes. It is also important to ascertain the distribution of bacterial contamination across the adjacent foreshore, in order to identify areas of potential high risk. Such information would highlight areas likely to be contaminated with other stormwater pollutants, as well as sites unsafe for harvesting of edible shellfish resources and areas unsuitable for shellfish restoration efforts.

Snelder and Williamson (1997) define stormwater as rainwater that runs off impervious surfaces and is usually discharged as a point source into waterways such as estuaries and harbors. When discharged into high-energy receiving environments there is immediate dilution, whereas sheltered habitats such as harbors and estuaries accumulate particulate matter contained in such discharges (Snelder and Williamson 1997). The main contaminants found in urban stormwater include heavy metals, polycyclic aromatic hydrocarbons (PAHs), organochlorine pesticides, hydrocarbons, nutrients, suspended solids, and microorganisms (Snelder and Truman 1995, Snelder and Williamson 1997). All have the potential to compromise the sustainability of marine environments (Morrisey 1997).

Stormwater discharges often affect the bacteriological quality of the receiving water (Gannon and Busse 1989). Of concern to environmental managers is the potential for microorganisms contained in waste discharges to affect human health adversely through bathing or the consumption of affected shellfish (Kebabjian 1994). Water and seafood contaminated with sewage have been reported to transmit gastrointestinal disorders, hepatitis, cholera, and a range of eye, ear, nose, and throat infections (McIntyre 1995). The spread of disease via contaminated water and seafood has prompted the use of indicator microorganisms as a warning of unsafe conditions (Elliot and Colwell 1985). These indicator microorganisms are not themselves pathogenic. They generally occur in high concentrations in the same environments as pathogenic microbes but are simpler to detect and quantify (Fattal et al. 1984). Therefore, they can be used to indicate a potential risk of disease (Elliot and Colwell 1985).

Survival of both pathogenic and indicator bacteria in marine waters is affected by many physical, chemical, and biological factors, including temperature, sunlight, salinity, predation, adsorption, sedimentation, and dilution (Borrego et al. 1983, Nicholson 1988). Biological parasitism and predation are also responsible for destruction of faecal bacteria entering the marine environment in untreated sewage (Roper and Marshall 1974). Survival characteristics of indicator bacteria and the pathogens they are modelling should be similar.

The coccoid bacteria *Enterococcus*, which naturally inhabits the gut of humans and warm-blooded animals, has gained favour in recent years over another bacterium, *Escherichia coli*, as an indicator bacteria, as they survive longer in seawater, and have good correlation with gastrointestinal symptoms (Miescier and Cabelli 1982, Elliot and Colwell 1985, Donnison 1992, Sinton et al. 1993).

Shellfish have the ability to concentrate bacteria and other contaminants from water or sediment via their mode of feeding, and are therefore useful tools for investigating faecal pollution (Ayres et al. 1978, Nicholson 1988, Prieur et al. 1990). Most bivalves are filter-feeders, passing large volumes of water across their gills to obtain food and oxygen. Microorganisms and food particles in suspension are trapped in mucus on gill, mantle, and labial palp surfaces and then transported by ciliary action to the mouth (Perkins et al. 1980, Cook 1991). Deposit-feeding bivalves feed by ingesting surficial sediment, which may have considerably higher levels of bacteria than the water column (Elliot and Colwell 1985, Kueh 1987). Accumulation of bacteria in sediments is due to their sorption to particles suspended in water, which then settle out onto the surficial sediment (Davies et al. 1995). Whether ingested via filter-feeding or deposit-feeding, digestive processes are not

thought to inactivate all microorganisms (Hedstrom and Lycke 1964). Depuration occurs through the discharge of faeces and from the pumping of water through the mantle cavity (Perkins et al. 1980). Factors affecting feeding, and therefore accumulation of bacteria, include temperature, turbidity, salinity, and physiological condition of shellfish (Bonadonna et al. 1990). Filtration rate may decline when these factors are suboptimal and therefore less indicator and pathogenic microorganisms would be accumulated (Ayres et al. 1978, Nicholson 1988). Retention of microorganisms by bivalves is a function of spacing of gill filaments, water flow through the mantle cavity, and filtering behavior, all of which add to the variability in accumulation of bacteria among species (Bonadonna et al. 1990).

This research aims to identify the effects of stormwater discharges on enterococci levels in bivalve shellfish, sediment, and water. Seasonal and year-to-year bacterial levels are investigated in experiment A, and event-driven episodes such as rainfall events are analyzed in experiment B. The spatial distribution of bacterial levels along the foreshore is presented in experiment C. This work uses relatively simple, inexpensive microbiological techniques to trace and determine spatial and temporal patterns associated with low volume waste discharges from small coastal communities into marine environments.

METHOD AND MATERIALS

Shellfish Description

Experiments in this study focus on the cockle, *Austrovenus stutchburyi* (Gray in Wood 1828), and the wedge shell, *Macomona liliana* (Iredale 1915). These two shellfish have overlapping distributions throughout many estuaries and harbors in New Zealand (Larcombe 1968, Marsden and Pilkington 1995). Both are abundant and widespread in the Whangateau Harbour. They have contrasting feeding modes (*A. stutchburyi* is a filter-feeder, and *M. liliana* is a deposit-feeder) and therefore different exposure routes to bacterial contamination.

Site Description

Three experiments were carried out in Whangateau Harbour, northeastern New Zealand (Fig 1). This harbor is typical of many New Zealand estuaries, and is fringed by several small, rural communities, with a combined population of c. 1400. Low levels of waste enter the harbor from these communities (e.g., Klein and Gowing 1993). Samples were taken from an area adjacent to a stormwater outfall at the putative impact site (Point Wells). This is a small residential community, of 324 people in 137 dwellings, with houses close to the foreshore and open stormwater drains. All homes have on-site sewage disposal systems (septic tanks). The comparison site at Lews Bay, across the harbor, has only three residences close to the shore.

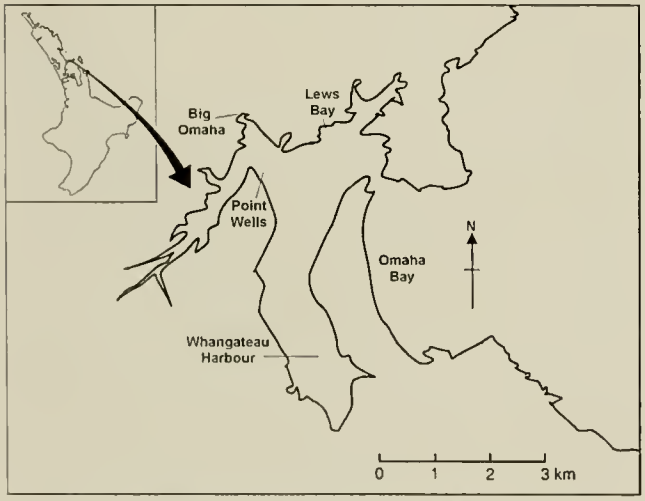

Figure 1. Location of sampling sites.

Sample Collection and Processing (All Three Experiments)

Shellfish

Bivalves were collected at low tide, placed in labelled plastic bags, and transported to the laboratory on ice, where they were processed within 6 h of collection. Shellfish were opened using aseptic techniques, and the whole animal was extracted and placed in sterile stomacher bags. For each sample, sufficient individual shellfish were used to provide a sample weight of between 10 and 40 g. Generally, 15 *A. stutchburyi* from both sites, 15 *M. liliana* from Lews Bay, and 7 *M. liliana* from Point Wells were used. Fewer *M. liliana* were required from Point Wells to achieve the desired sample weight, as they were larger than those from Lews Bay. Bags containing samples were individually placed in a Seaward Stomacher laboratory blender (model 400) and macerated at normal speed for 2 min. Samples were diluted tenfold with Geldreich phosphate buffer (WHO 1982). A five-tube Most Probable Number (MPN) series of azide dextose broth, as described by Donnison (1992), was set up, with double-strength medium used in the first row. Tubes were inoculated with 10, 1, and 0.1 ml of diluted sample, according to the MPN series, and then incubated in water baths at 35°C for 48 h. Positive tubes (turbid) were plated onto membrane filters, which had been divided into six segments (one for each of the five tubes within a dilution and one control segment). Filters were placed onto mE agar plates, which had been brought to room temperature. Plates were inverted, placed in a sealed plastic box and incubated for 48 h at 41 ± 0.5 °C. Filters were aseptically transferred to esculin iron agar (EIA) plates (which had been equilibrated at room temperature), and incubated at 41 ± 0.5 °C for approximately 20 min. Streaks of growth that were pink-to-red with a brownish-black precipitate underneath were scored as positive. MPN scores were determined from standard MPN tables and bacteria per 100 g of shellfish tissue calculated.

Sediment

Surficial sediment samples (approximately 50 g) were collected by scraping a sterile stainless steel scoop across the sediment at low tide, capturing the top centimeter of sediment. The scoop was washed and flamed in alcohol prior to the collection of each sample in order to ensure no cross-contamination. Sediment was placed in sterile plastic bags and transported on ice to the laboratory and processed within 6 h of collection.

Sediment samples were prepared according to the enterococci MPN technique described by Donnison (1992). Approximately 10 g of each sample were weighed out and placed in a sterile plastic bag. A tenfold dilution was made using sterile phosphate buffered

diluent (WHO 1982). Diluted samples were shaken by hand for 2 min to release bacteria from the sediment into suspension. Diluted samples were then used as the inoculum for a five-tube MPN series and processed as for shellfish.

Water

Water samples were collected in sterile glass bottles and transported to the laboratory on ice and away from sunlight. The mE/EIA membrane filter technique was used to enumerate enterococcus concentration (APHA 1992, Donnison 1992). Three replicates of 10 ml and three replicates of 50 ml from each water sample were filtered through a Sartorius 0.45-μm cellulose nitrate filter paper, which was aseptically transferred to mE agar plates (previously equilibrated at room temperature). The plates were inverted, placed in a sealed plastic box, and incubated for 48 hours at 41 ± 0.5 °C. Filter papers from plates showing positive growth were transferred to EIA agar plates and incubated for a further 30 min. Colonies that were pink-to-red with a brownish-black precipitate underneath were scored as enterococcus. The mean number of colonies per 100 ml of sample was calculated.

Rainfall data were derived from daily information collected at the University of Auckland, Leigh Marine Laboratory, approximately 15 km from sampling sites.

Experimental Protocols

Experiment A

The first experiment investigated the seasonal and year-to-year variability of enterococci levels in the body tissue of cockles and wedge shells. Three replicate composite samples were collected from each site, 50 m from the foreshore, every 2 mo during 1996–1998, and analyzed according to the schedule above. Median MPN of enterococci per 100 g shellfish flesh were plotted against maximum daily rainfall over the preceding 4 days.

Experiment B

The long-term monitoring in experiment A revealed high variability in enterococci levels, and peaks of enterococci appeared to be related to high rainfall. This hypothesis was examined in more detail in this second experiment, which investigated the effect of specific rainfall events on the microbiological quality of shellfish tissue, sediment, and water. Samples were taken from late April to early May for a 14-day period in 1996 and a 12-day period in 1997. Each day at low tide a single sample each of sediment, *A. stutchburyi*, and *M. liliana* was taken from the sites at Point Wells and Lews Bay. Sampling sites were 50 m from the foreshore at both sites. A stormwater sample and a harbor water sample (from Big Omaha Wharf, see Fig. 1) were also collected daily. Processing and analyses were carried out as detailed above. Median number of enterococci/100 ml water were plotted against daily rainfall and compared with MPN enterococci/100g of shellfish flesh and sediment. Median enterococci concentrations for harbor water, sediment, and both shellfish species were calculated using data from all experiments (A, B, and C) and plotted with results of this experiment (B) to provide a comparison with typical levels.

Experiment C

Data from experiment B regarding the temporal patterns of bacteria in shellfish and sediment prompted investigation into the spatial patterns of bacterial levels in sediment and tissue of *A. stutchburyi* around a stormwater outfall at Point Wells. To delineate the effects a suite of experiments was carried out using three 80-m transects extending seaward from the stormwater drain outfall. The first transect was placed perpendicular to the foreshore. The other two transects were placed on either side of the first transect, at 45° and 135° to the foreshore (see Fig. 2). *Austrovenus stutchburyi* samples were taken in July and August 1998 every 10 m from 10 to 80 m along the central transect and from 20 to 80 m along the two radials. In July 1998, sediment samples were taken at 0 m, 5 m, and then every 10 m out to 60 m on the central transect, and 2.5 m, 5 m, and every 10 m out to 60 m on the radial transects. MPN enterococci/100g shellfish flesh and sediment were plotted against distance from the stormwater drain.

RESULTS

Long-term temporal variability in enterococci levels in *A. stutchburyi* and *M. liliana* tissue plotted against maximum daily rainfall for the preceding 4 days is shown in Fig. 3 a and b. Bacterial levels of 20 MPN/100g were at or below the detection limit of the procedure, which is shown as a horizontal dotted line on each graph. Peaks in the levels of enterococci occurred over the winter months (June to October) (Fig. 3 a and b), and these winter peaks approximately corresponded with highest rainfall. Bacterial levels were also raised in February and April for *A. stutchburyi* at Point Wells. Only three results over the entire sampling period at this site for *A. stutchburyi* were below the detection limit of 20 MPN/100g. Bacterial levels were below detection in most months for *A. stutchburyi* at Lews Bay, and for *M. liliana* at both sites (Fig. 3 a and b). During the sampling program, enterococci levels in *A. stutchburyi* were above the detection limit 77% of the time at Point Wells, compared with 24% at Lews Bay. The detection limit was exceeded 41% of the time for *M. liliana* at both sites.

The effect of a rainfall event on stormwater and harbor water quality was specifically investigated in the first period of the second experiment. During intense monitoring in the autumn of 1996, rainfall of 40 mm on day 5 resulted in an approximate 100-fold increase in enterococci on day 5 for stormwater and on day 6 for harbor water (Fig. 4a). Enterococci levels then declined and returned to background levels (< 100/100 ml) by day 8. Enterococci

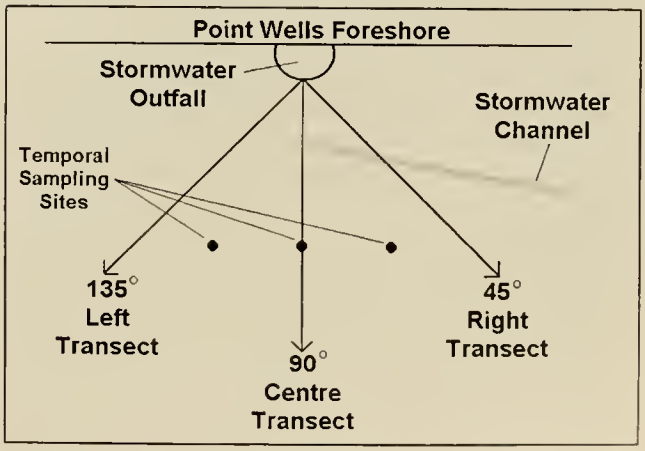

Figure 2. Position of temporal shellfish and sediment sampling sites and transect design for spatial sampling around stormwater outfall at Point Wells.

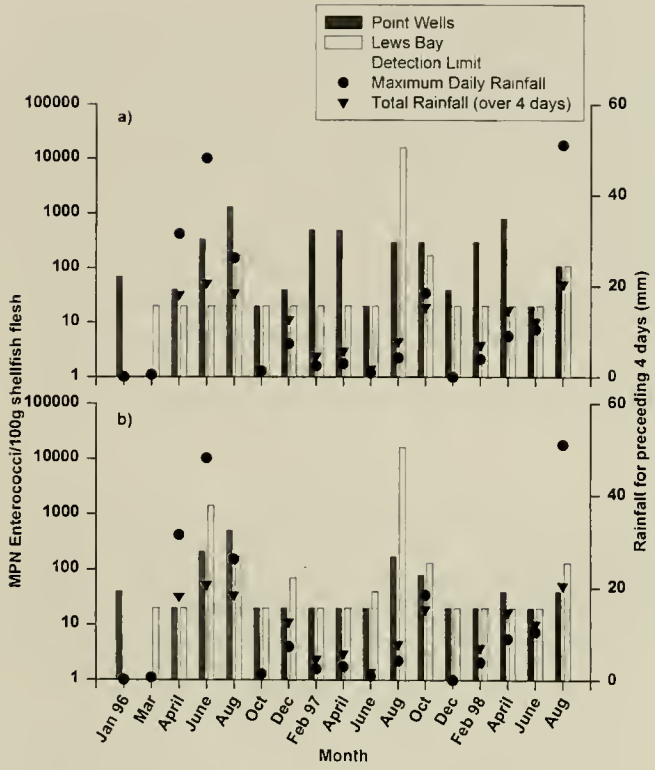

Figure 3. Temporal pattern of enterococci levels in body tissue of (a) *A. stutchburyi* and (b) *M. liliana* at Point Wells and Lews Bay.

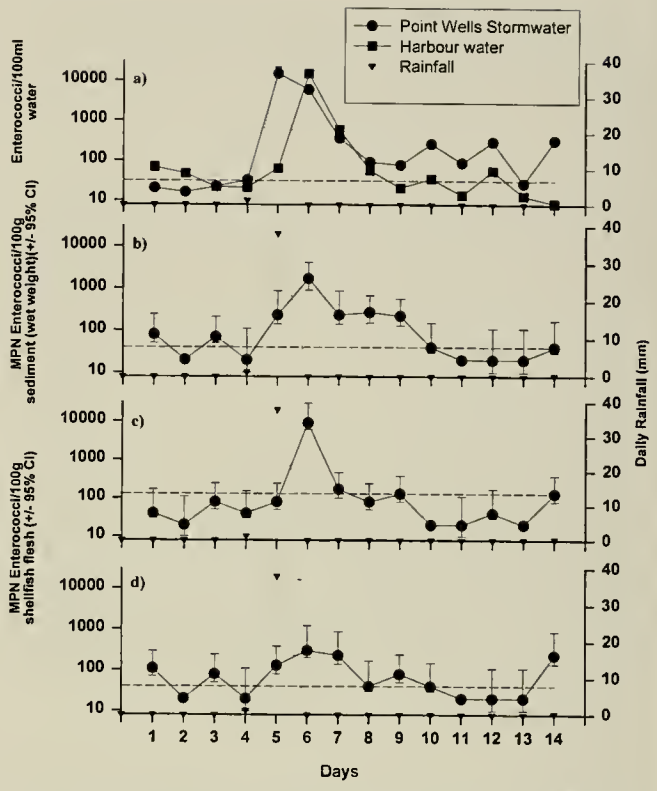

Figure 4. Daily enterococci levels over 14 days in April and May 1996 in (a) stormwater and harbor water, (b) sediment, (c) *A. stutchburyi*, and (d) *M. liliana* tissue. Median (background) enterococci levels are shown as a dashed line.

levels in sediment showed a similar pattern (Fig. 4b), with increased concentration on day 6 (the day after the rainfall event) to approximately 1000 MPN/100g, followed by a slower decline over the subsequent few days to background levels of less than 100 MPN/100g. Analyses of tissue from *A. stutchburyi* showed the same pattern (Fig. 4c), reflecting harbor water quality at 10,000 MPN/100g on day 6. Bacterial depuration by this bivalve was very rapid, with levels declining to approximately 100 MPN/100g by day 7. *Macomona liliana* were affected to a lesser extent, with a slight rise above 100 MPN/100g on days 6 and 7, remaining elevated until day 8 (Fig. 4d). These patterns suggest that enterococci levels in *A. stutchburyi* closely reflect the bacterial levels in water, whereas those for *M. liliana* more closely mimic levels in sediment.

The second intensive monitoring period, in 1997, was during a period where rainfall did not exceed 4 mm on any day. Enterococci levels in stormwater and harbor water were not above 100 MPN/100ml during the sampling period, with a small rise above background levels in harbor water due to factors other than rainfall (Fig. 5a). Levels in sediment did not rise above 100 MPN/100g and were only marginally higher than median background levels at any time (Fig. 5b). Bacterial levels in *A. stutchburyi* were more variable, with most results ranging between 100 and 1000 MPN/100g (Fig. 5c), often above background levels. Again, enterococci levels remained relatively constant at or below 100 MPN/100g for *M. liliana*, with small increases above background levels occurring (Fig. 5d).

The first in the suite of experiments investigating the spatial distribution of enterococci around a stormwater outfall at Point Wells was carried out after 4 days of very heavy rainfall (daily maximum 118.9 mm) in July 1998. There was little change in bacterial levels with increased distance from the origin (i.e., the stormwater drain) (Fig. 6 a–c). Along the central transect enterococci density declined from 300 MPN/100g at 0 m to 70 MPN/100 g at 70 m (Fig. 6a). A similar pattern is evident along the two radial transects, with levels dropping from 230 to 270 MPN/100 g at 20 m to 20–90 MPN/100 g at 80 m respectively (Fig. 6b and c). This experiment was repeated during a moderate rainfall event in August 1998; the maximum daily rainfall for the preceding 4 days was 13.2 mm. The overall pattern was different on this occasion, with levels nearest to the origin not being the maximum for each transect (Fig. 7a–c). Results for the left-hand transect (Fig. 7b), which is directed away from the flow of the stormwater stream (see Fig. 2), showed a relatively clear pattern of lower bacterial levels with increased distance from the stormwater outfall. However, bacterial levels along the right-hand transect reached a maximum at 30 m (Fig. 7c), which is the point where the stormwater stream crosses this transect (Fig. 2). Levels are high for the full extent of this transect. The central transect's lowest bacterial levels are at the origin and highest levels at the 80 m mark, with high variability among points (Fig. 7a).

Enterococci levels in sediment declined from 9000 MPN/100g at 0 m to 270 MPN/100g at 60 m along the central transect (Fig. 8a). The pattern is similar for the left-hand transect (Fig. 8b), although the first sample at 2.5 m (1100 MPN/100 g) is lower than that at 5 m (3000 MPN/100 g). However, the density of enterococci declines to 340 MPN/100 g at 60 m. Figure 8c indicates a decline in bacterial levels with increased distance. Daily maximum rainfall for the preceding 4 days was 46.3 mm.

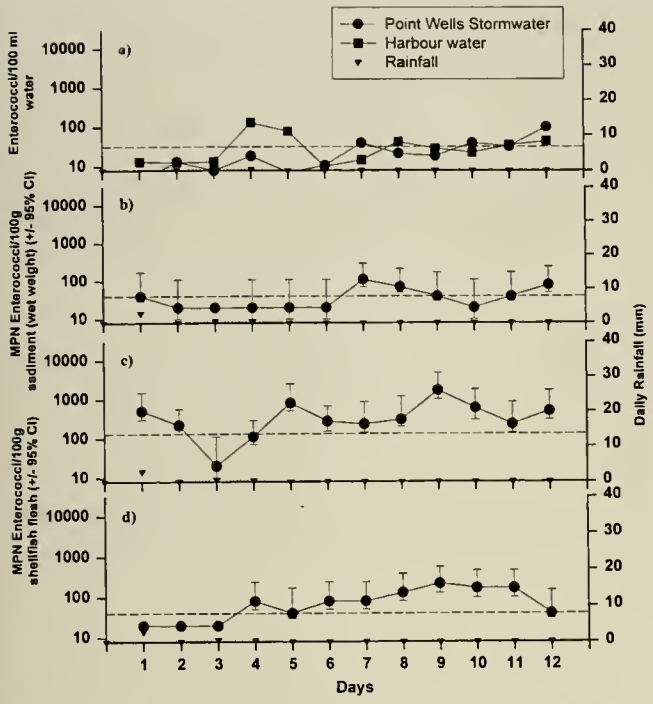

Figure 5. Daily enterococci levels over 12 days in April and May 1997 in (a) stormwater and harbor water, (b) sediment, (c) *A. stutchburyi*, and (d) *M. liliana* tissue. Median (background) enterococci levels are shown as a dashed line.

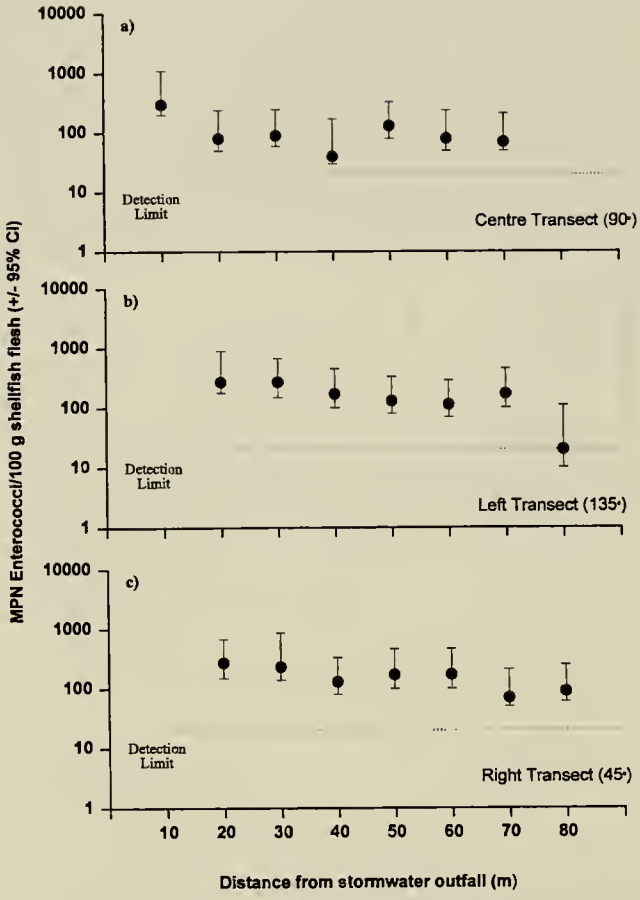

Figure 6. Enterococci levels in *A. stutchburyi* tissue around stormwater outfall at Point Wells following heavy rainfall (July 27, 1998).

DISCUSSION

There is a seasonal nature to the patterns of enterococci levels in shellfish in the Whangateau Harbour. As both species at both sites exhibited peaks during the wetter winter months, it is likely that these increases in bacterial levels are due to the cumulative effects of many stormwater outfalls, nonpoint source runoff, and leaching from on-site sewage systems. Soils often become saturated during winter months due to high rainfalls and flooded effluent leach fields associated with on-site wastewater treatment can cause the discharge of untreated wastewater into the marine environment (Gover 1993). Other researchers have found similar seasonal effects. For example, Paille et al. (1987) detected a peak in enterococci levels in oysters in Louisiana during late spring-early summer plus a peak in early winter. However, as no rainfall figures are given, it is difficult to ascertain the cause. LeMay et al. (1995) found that in the first few months of winter, when there was high rainfall, the levels of enterococci in marine water samples in California were correspondingly high. Levels dropped to a background level of around 100 Colony Forming Units (CFU)/100 ml in the later winter months.

A confounding factor in winter is decreased water temperature, as lower temperatures generally affect survival and detection of bacteria (Ayres et al. 1978). Inhibition due to sunlight can also decrease bacterial survivorship in summer (Borrego et al. 1983). *Austrovenus stutchburyi* at Point Wells exhibited high enterococci levels in winter and summer months, and it is likely that *A. stutchburyi* at this site have higher background levels overall. *Macomona liliana* at Point Wells did not exhibit high levels in summer, and this may be related to both their location 10–15 cm below the sediment surface, where they are more removed from waste discharge flows, and to different feeding methods. The summer enterococci peak detected in *A. stutchburyi* may be due to the influx of holiday visitors to this coastal settlement during the warmer months, which would place a strain on the ability of on-site sewage systems to adequately treat wastewater. This may lead to leach fields becoming overloaded and surface or groundwater seepage into the open stormwater drains or directly into the harbor. It can be concluded that factors other than simply winter rainfall affect the microbiological quality of cockles at Point Wells. Further, in terms of human health risk, wet summers, where high rainfall and increased human population occur concurrently, may represent the "worst case" scenario. The experiments that investigated the effect of rainfall events on water, sediment, and shellfish bacterial levels unequivocally indicated that rainfall is associated with increased levels of enterococci. This immediate effect on enterococci levels in stormwater with increased rainfall (Fig. 4a–d) is intuitive, as stormwater is defined as rainwater directed from impervious surfaces and stormwater is known to generally contain high levels of fecal bacteria. Indeed, Pitman (1995) found > 10,000 fecal bacteria per 100 ml in stormwater runoff samples taken in Goleta, California. The delayed effect on the harbor waters found during our study is likely to be due to dilution of the input from many stormwater drains and runoff from adjacent roads and farmlands, with the cumulative effect not being detected until the day following the rain event. However, LeMay et al. (1995) concluded that during wet weather levels of enterococci in marine waters increased dramatically and subsequently fell to background levels within 2–3 days. The one-day delay in accumulation of bacteria by *A. stutchburyi*, *M. liliana*, and sediment is also expected, as shellfish would

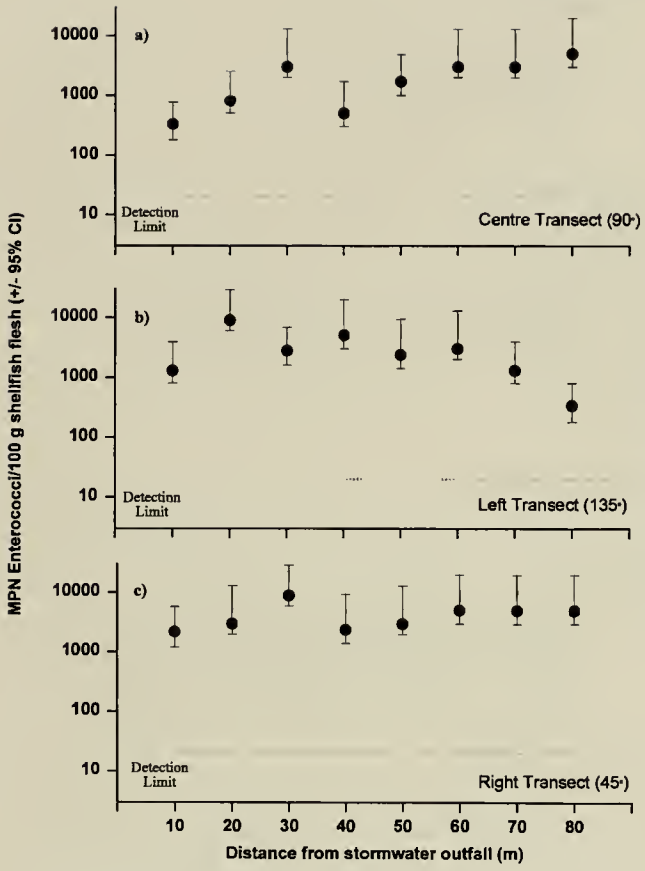

Figure 7. Enterococci levels in *A. stutchburyi* tissue around stormwater outfall at Point Wells following moderate rainfall (August 28, 1998).

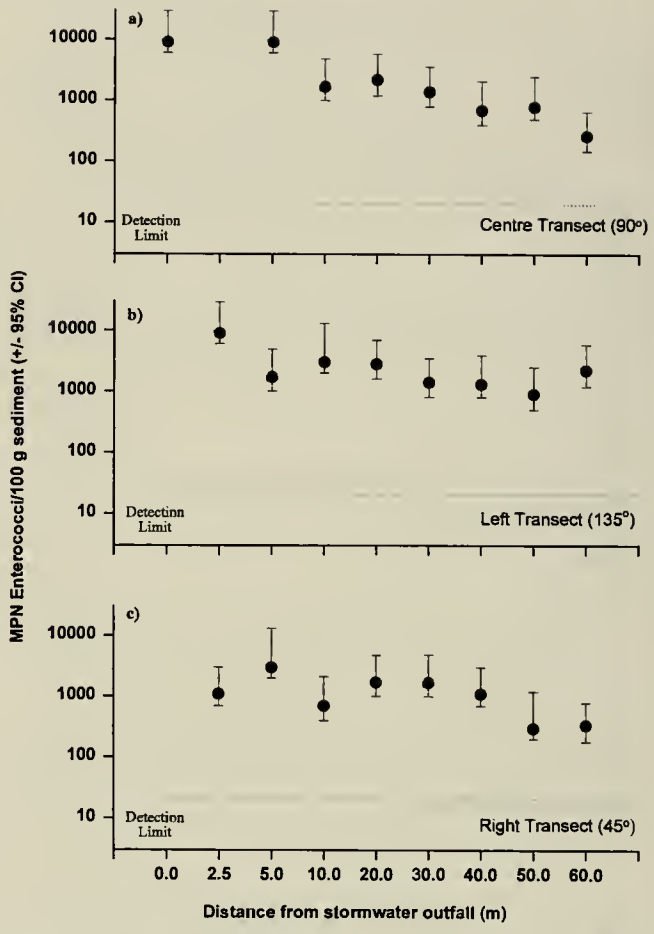

Figure 8. Enterococci levels in surficial sediment around stormwater outfall at Point Wells (July 13, 1998).

take at least one tidal cycle to accumulate high levels of bacteria, and as sediment samples were taken at low tide, it is unlikely that bacteria from the stormwater outfall would have sorbed to surficial sediment sampled on day one. Similar increases in bacterial levels following heavy rainfall have been found in sediments (Goyal et al. 1977) and shellfish (Paille et al. 1987, Pitman 1995).

Samples taken during a 12-day period of little or no rainfall give an indication of the background variability in enterococci levels for *A. stutchburyi* in the Whangateau Harbour (Fig. 5c). It is clear that *A. stutchburyi* shows greater variability in enterococci levels than *M. liliana*, and this could be due to its proximity to the sediment surface (and therefore the discharge flow) and filter-feeding mechanism.

The delineation experiments (Figs. 6–8) have complex interpretations. Transects sampled after very heavy rainfall (Fig. 6) give the anticipated pattern of decreased enterococci levels with increased distance from the stormwater outfall. However, the levels overall are relatively low, and this is likely to be due to the flushing effect of high rainfall (LeMay et al. 1995). After a more moderate rainfall event (an order of magnitude lower than the peak levels experienced) the pattern is unclear. Enterococci levels along the central transect increase to a maximum at 70–80 m, and this may be due to pooling of water in this area, which can cause bacteria to be retained in water from which cockles feed. The left transect, extending away from the direction of the stormwater channel shows the more expected decline in bacterial levels with increased distance from source. Enterococci levels are high for the length of the right transect, and at the 30 m mark, due to the transect crossing the stormwater channel, an increase is detected. It is likely that levels remain high along this transect due to its proximity to the stormwater channel, which overflows onto the adjacent "flood-plains" and crosses the path of the transect.

Enterococci in sediment decline in an anticipated almost linear pattern with increased distance along the central and right transect (Fig. 8). Again the pattern along the left transect is different, with distant samples also having high levels. The cause is likely to be overflow from the stormwater channel at low tide, as the daily maximum rainfall for the 4 days prior to this particular sampling exercise was high (46 mm).

The identification of ecological impacts in harbors and estuaries is problematic due to natural temporal and spatial variability in populations (Snelder and Williamson 1997). A better approach may be to focus on stormwater treatment rather than identification of effects. In New Zealand, mitigation of effects of stormwater discharges is based on the "Best Practicable Option" as defined by the Resource Management Act 1991. Treatment generally involves either sedimentation in ponds or filtration, both of which still allow some contaminants to enter the receiving environment (Snelder and Williamson 1997). However, Treworgy and Garrett (1989) believe loss of coastal resources due to effects of waste discharges often must incur a financial hardship in order to justify restoration costs. A negative impact on the economy of the Ria Formosa area in Portugal was attributed to an increase in anthropogenic discharges and the associated decrease in bivalve production (Bebianno 1995). However, it is generally very difficult to calculate the

financial cost of contaminated marine environments, and it may be this factor that hinders remediation and mitigation processes.

We conclude that enterococci can be used to trace the spatial and temporal extent of stormwater discharges from small, urban coastal communities. This information, once combined with knowledge of the common toxic contaminants in such discharges, can be used to identify coastal areas suitable for shellfish restoration programmes, as well as detect and model potential effects on the marine environment and shellfish resources.

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RED AND GREEN ABALONE SEED GROWOUT FOR RESEEDING ACTIVITIES OFF POINT LOMA, CALIFORNIA

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ABSTRACT California has a statewide closure on harvest of any abalone species from San Diego County north to San Francisco. Prior to closure, harvests of the main species that compose the fishery (the red abalone, *Haliotis rufescens* Swainson 1822; the green abalone, *Haliotis fulgens* Philippi 1845; and the pink abalone, *Haliotis corrugata* Gray 1828) had plummeted to an all-time low of several hundred thousand pounds per year. Overfishing and several diseases identified recently in abalone are creating concern within the industry with respect to the recovery of the fishery. One method being considered to restore the fishery on a limited basis is reseeding. Reseeding success is dependent on many factors, including planting location and techniques of seed placement. Other important factors include the abundance and nutritive quality of macroalgae (kelp) available to the abalone, the seawater temperature requirement of the species being seeded, the size of the seed being planted, and the presence of potential predators within the seed site. In this study, we observed that shell growth and weight gain in juvenile red and green abalone are temperature-dependent. When constant, elevated temperatures are maintained, growth rates improved as compared to growth rates in animals kept at the lower temperatures normally found. More growth in juvenile red abalone was observed at 19 °C, with an average daily shell growth of 0.1 mm/day. Previous work suggested that *Egregia laevigata*, a brown kelp was a superior food source for juvenile abalone; however, the growth rates of red abalone fed *Egregia* increased minimally compared to growth rates of juvenile red abalone fed another brown kelp *Macrocystis pyrifera*. Maximum sustained shell growth of 0.16 mm/day in juvenile green abalone was observed at a culture temperature of 21 °C. Extrapolations of these growth rates for an entire year indicate growth of 36 mm/y for red abalone and 60 mm/y for green abalone. Growth predictions based on laboratory-reared juveniles will vary from those observed in wild populations since seawater temperatures are not static along the coast and may vary as much as 10–12 °C during the year. The nutritional value of kelp and other algae may be as important as temperature in the effect on growth observed in the laboratory and in the field. Increased growth rates exhibited by juvenile red abalone during the period from January through April 1977 may be reflective of changing nutrient ratios or levels in kelp that are otherwise limiting during reduced or non-upwelling months. We are presently culturing hatchery-produced red and green abalone seed for eventual planting in the U.S. Department of the Interior's Cabrillo National Monument. The seed are approximately 45 mm in size and are fed a mixed kelp diet. They will be transplanted when they are approximately 75–100 mm in size. Reseeding success assessed by annual dive surveys should help determine if planting larger animals increases survival of brood stock and increases recruitment within the monument area.

KEY WORDS: Green abalone, red abalone, reseeding, growout, growth rates

INTRODUCTION

Historical Trends of the California Abalone Fishery

The present day abalone fishery evolved from the intensive harvesting activities of Chinese immigrants in the 1850s (Cox 1960). Abalone is a revered food item in the Orient, and the Chinese in California had developed a substantial fishery by 1879 based upon the intertidal green abalone, *Haliotis fulgens*, and the black abalone, *H. cracherodii*. Commercial landings in California at that time exceeded 4.1 million pounds (total weight equals shell and meat weights combined) (Cox 1960). Soon thereafter, the Japanese "sake barrel" divers replaced the Chinese-dominated fishery, followed by the "hard hat" divers. This change in harvesting methods (pumping surface air to submerged divers via air hoses) permitted harvesters to maintain landings while operating in deeper offshore waters. After 1916, three major trends were observed within the fishery: (1) a locational shift of the fishery center from Monterey south to Morro Bay, Santa Barbara, and Los Angeles; (2) a compositional change in the species of abalone harvested for the fishery; and (3) a drastic decline in total abalone landings after 1965 (Cicin-Sain et al. 1977).

Long-term trends of the fishery in the state depict an abrupt increase in landings from 146,462 pounds in 1942 to 4,784,033 pounds in 1952 and then a leveling off in total landings until 1966 (Heiman and Carlisle 1970). The fishery flourished between 1958 and 1968, with average annual landings in excess of 4.5 million pounds. Since 1968, however, landings have decreased. Only 1.3 million pounds were taken in 1973. Landings in the state in 1994 totaled only 322,000 pounds or 6% of that recorded 30 years ago (Fig. 1). The decline in landings have been attributed to intense harvesting procedures by commercial and sport divers, environmental degradation of habitat, predation by sea otters, and in some cases, competition for space and food from sea urchins (Cicin-Sain et al. 1977, Tegner et al. 1981).

Recent Strategy

Reduced yields for both the commercial and sport fisheries have prompted more stringent management regulations and have stimulated interest in developing methods for enhancing natural populations. This involved limited entry to the commercial fishery and more stringent limitations on sport fisherman; however, landings still continued to decline. Most recently, the commercial and recreational abalone fishery, south of San Francisco, was closed in August 1997 so that a fishery management plan could be developed. Recent work appears to indicate that the dispersal of abalone larvae may not be as widespread as once thought, indi-

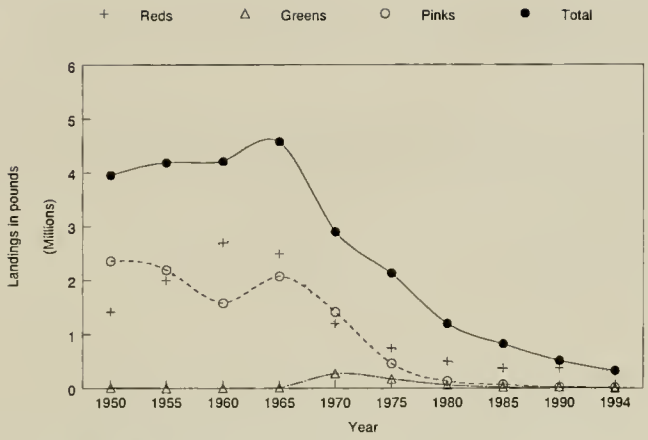

Figure 1. Commercial landings of abalone by species in California for the years 1954–1994.

cating that closure of a depleted fishing ground may not be sufficient for recovery to occur. Our strategy for recovery emphasizes replenishing previously depleted fishing areas by release of hatchery-reared juveniles and establishing populations in presently unutilized or artificially improved habitats (Tegner et al.1981, Ebert and Houk 1984, Tegner and Butler 1985, Schiel 1993, Kojima 1995).

Factors Affecting Abalone Growth

Reseeding success is dependent on many factors, including the geographic location for the placement of seed and techniques of seed placement. Other factors include the abundance of kelp, the temperature requirement of the species being seeded, the size of the seed or juveniles being planted, and the presence of potential predators within the site. Growth rate data from earlier work and this study on the red abalone and the green abalone as a function of culture temperature and diet were reviewed. That is, what length of time is required to grow larger (76–89 mm in size) *H. rufescens* (Fig. 2) and *H. fulgens* (Fig. 3) for field reseeding? A review of previous research on growth rates in several species of abalone indicated extreme variability related to age, species, food supply, environmental conditions, geographic location, and season.

Tutschulte (1976) described growth rates for juvenile pink abalone, *Haliotis corrugata*, green abalone, *H. fulgens*, and white abalone, *H. sorenseni*. Juveniles of each were cultured in the laboratory, reared in seabed cages, and marked and released into kelp beds. The annual increase in shell length for all three species ranged from 10.7 to 22.6 mm in the laboratory, 10.7 to 17.3 mm in seabed cages, and 20.4 to 29.2 mm in the wild. An annual increase in shell length of 25.9 mm was measured for laboratory-reared juvenile red abalone, *H. rufescens* (Leighton 1968). However, growth rates of young *H. rufescens* held in seabed cages off La Jolla showed marked variation, with annual increases in shell length from 9.8 to 35 mm. Similarly, juvenile red, pink, green, and white abalones exhibited large variations in growth as a function of seawater temperature (Leighton 1972, 1974). Variation in growth rates of juvenile abalone has been observed in *H. discus hannai* (Sakai 1962), *H. tuberculata* (Foster 1967), and *H. midae* (Newman 1968). Clearly, laboratory conditions must be strictly controlled and monitored if the growth rate of a particular species of abalone is to be determined accurately.

Other studies indicated that growth in abalones was dependent upon temperature (Sakai 1962, Leighton and Boolootian 1963, Tomita and Saito 1966, Leighton 1972, 1974; McBeth 1972, Lapota 1978, 1982), and perhaps to a lesser extent on the type and quality of the diet (Kikuchi et al. 1967, Leighton 1968, 1976, Leighton and Boolootian 1963, Tomita 1972). Our study examined the influence of temperature and diet on the growth of juvenile red and green abalones.

Previous studies seem to indicate that the release of larger abalone (>25 mm) may substantially increase the chance of maintaining and enlarging the number of brood stock for later recruitment (Inoue 1976, Tegner and Butler 1985). Larger abalone are less cryptic than smaller seed, which will make survival assessment easier, and they also have thicker shells, which offer more protection from predation by crabs, lobsters, and octupuses.

MATERIALS AND METHODS

Culture System: Hubbs—Sea World Research Institute

Two flow-through seawater culture systems were used to culture both red and green abalones. In an earlier study in 1978, conducted at the Hubbs–Sea World Research Institute (HSWRI) (Lapota 1982), research was conducted in a seawater laboratory equipped with automatic filtration and large heat exchanger systems. Three fiberglass tanks were modified and divided into six water-bath compartments (Fig. 4). Tank dimensions were 241 cm long, 73 cm wide, and 76 cm high. All three tanks were mounted on a wood platform to insulate the bottom of the tanks and allow for plumbing of seawater supply and drainage. The main wall partitions of each tank were reinforced fiberglass wood cells 12 cm thick. An aisle was left between the tanks to provide for access during maintenance and survey activities. Polyethylene containers (13-L volume) used to confine and culture the abalones were placed on concrete bricks in each compartment of the water baths. The containers were fitted with four drain fittings positioned equidistant around the circumference above the height of the water in the controlled temperature bath. A single water input jet was placed in the bottom of each container and powered by an air stone to produce air lift for circulation. Water was introduced into each container through the bottom, and returned to the controlled temperature bath through the drain fittings at the top. Seawater was added to each water bath at a rate of 0.3 L/min, which gave an exchange rate of once per day. Seawater was supplied to each tank through a 5 cm I.D. PVC pipe manifold on the center wall partition of each tank. Excess seawater was drained through the tank overflow in the controlled temperature bath.

Ambient seawater temperatures ranged from 15 to 19 °C at Perez Cove in Mission Bay, San Diego, during the study period, and had to be adjusted to the prescribed culture temperatures. To maintain the controlled temperature baths at 13, 15, and 17 °C, ambient temperature seawater was cooled by circulating chilled fresh water from a cold sump through plastic heat exchange coils (16 mm O.D. by 13 mm I.D.) immersed in each of the three controlled temperature baths. Magnetic drive pumps powered by thermostatic controllers delivered the cold water to the coils as required. A refrigeration unit was used to keep the cold freshwater sump at 4 to 7 °C. The controlled temperature bath of 19 °C was heated with a 1000-watt immersion heater during the months when seawater temperature was cooler.

Figure 2. The red abalone, *Haliotis rufescens*. Approximate size is 3.8 cm. Photo by Jaelyn Chock.

Figure 3. The green abalone, *Haliotis fulgens*. Approximate size is 3.8 cm. Photo by Jaelyn Chock.

Culture System: Space and Naval Warfare Systems Center, San Diego

The Biological Effects Program (BEP) bioassay facility in Point Loma is the current location for abalone seed growout activities (Fig. 5). The BEP was established to test new experimental ship hull coatings proposed for use in the U.S. Navy as well as to evaluate sediment for contamination in San Diego Bay. The facility also houses the portable microcosms for environmental testing (POMFRET); these microcosms can be deployed on-site to evaluate the chronic effects of pollutants on resident marine organisms (Henderson 1990). The core of the system consists of 12 outdoor 128-L tanks (Fig. 5). The tanks are designed for semi-continuous, flow-through of unfiltered seawater and are exposed to sunlight. POMFRET flow control is accomplished with a "batch delivery" system. A series of paired, adjustable-volume bins are periodically filled with ambient water and are automatically emptied by activation of cap valves cycled by an adjustable-speed motor. Supply water for the POMFRET system is pumped from San Diego Bay near the entrance to the bay by a pair of 3 horsepower swimming pool pumps. Only one pump is used during normal operation; the other pump is on standby. Operation of pumps is normally alternated biweekly to allow cleaning of the inactive pump's intake and output plumbing (Henderson 1990). A gravity supply tank provides constant flow of ambient water to a feed tank (approximately 57-L volume), excess water overflows into a standpipe. Overflow from each of the POMFRET's 12 abalone tanks and four other larger tanks is fed into the City of San Diego's sewer system. All tanks are continuously aerated.

Culture System: Naval Ocean System Center, San Diego

Earlier investigations at the Naval Ocean System Center in Point Loma (now known as SPAWAR Systems Center, San Diego) from September 1976 through July 1977 were conducted to determine the temperature that would promote the most rapid growth in the red abalone. A culture system was developed that permitted accurate temperature control. Several groups of 60-L culture aquaria were used as temperature baths. Two 13-L polyethylene containers in each aquarium confined the experimental groups. Concrete bricks in each container provided a substrate for juveniles. Seawater was continuously aerated and changed twice a week. One hundred twenty hatchery-reared red abalone, ranging from 12 to 15 mm in shell length, were purchased from Ab Lab at Port Hueneme, California. All were maintained on a diet of *Macrocystis pyrifera*. These animals were cultured at a series of temperature in the range 16.5–22 °C. Shell lengths and total weights of each juvenile were recorded every 30–35 days.

In a larger study conducted at HSWRI, hatchery-reared juvenile red abalone were cultured for 175 days at constant temperatures of 13, 15, 17, and 19 °C. The juveniles, averaging 10–12 mm in length, were purchased from California Marine Associates, Cayucos, California. The abalones were divided into eight test populations, two per temperature bath. Each group was fed *ad libitum* with either the giant kelp, *Macrocystis*, or the feather-boa kelp, *Egregia*, twice weekly throughout the entire study. Preliminary results on *H. rufescens* showed a seasonal response with peaks in growth rates in late winter and spring, possibly caused by the increased nutritive value of the giant kelp, *M. pyrifera*. Therefore, to investigate dietary quality-temperature effects, hatchery-reared juvenile red abalones were fed on a diet of either kelp at constant temperatures. A parallel study examined growth of wild-caught juvenile green abalone at constant and warmer temperatures of 17, 19, 21, and 23 °C. The initial mean size of the green abalones was approximately 30 mm. The feeding schedule was the same as in the study of red abalone. Shell length (to 0.1 mm) and blotted wet weight (to 0.1 gm) of each abalone were measured at 35-day intervals.

In the later study conducted at SPAWAR Systems Center, 35 hatchery-reared green abalones were grown for 15 mo at ambient seawater temperatures. The mean starting size of the green abalones was 22 mm. Measurements were conducted sporadically except for the fall-winter measurements (1997–1998) which were made on 30–40 day increments.

RESULTS

First Data—Nutritional Observations (SPAWAR)

Data on juvenile *H. rufescens* shell growth were obtained for a period of 317 days (Fig. 6). Distinct increases in shell growth were observed at all culture temperatures from late January through

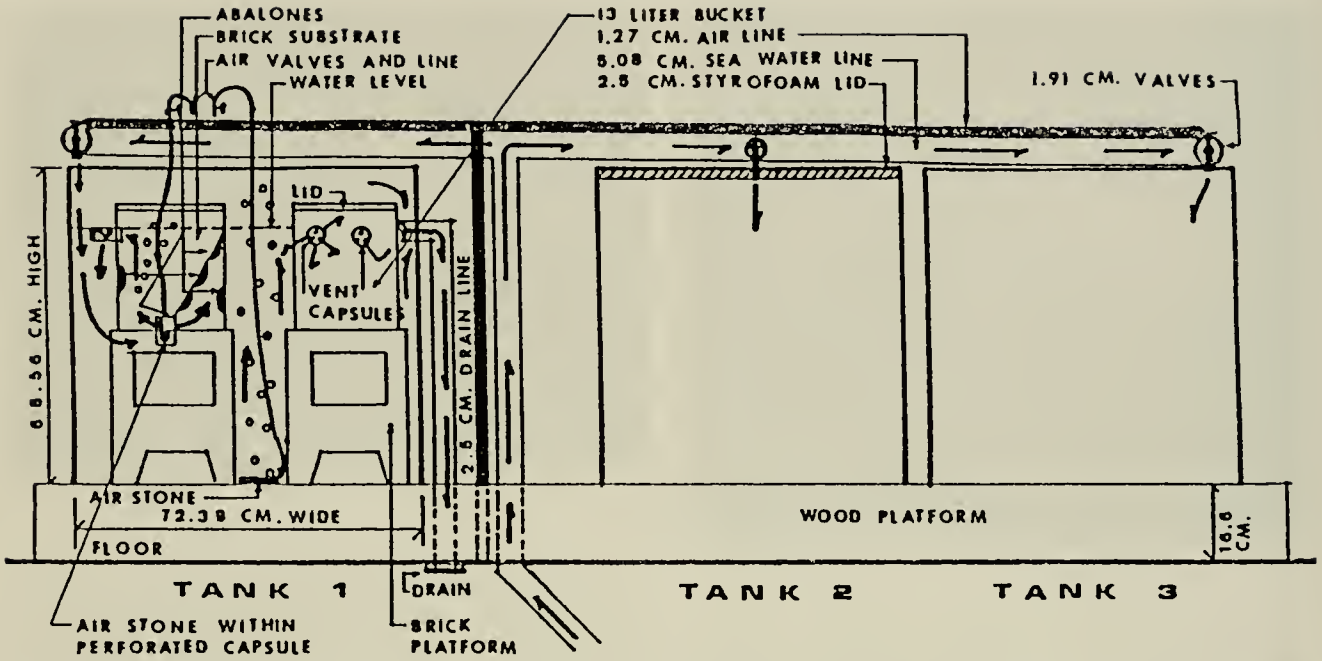

Figure 4. End view of tanks and schematic of water circulation through abalone culture system used at the Hubbs-Sea World Research Institute, Mission Bay, San Diego, California.

April 1977. Shell growth after April declined noticeably at all four temperatures, and was reduced markedly from late September through early January. Clearly, growth of juvenile *H. rufescens* was maximum in late winter and early spring at all temperatures tested. However, the groups cultured at 16.5 and 17 °C grew faster than those at 20 and 22 °C.

Constant Seawater Temperature Culture (HSWRI)

The mean starting size of the red abalones grown at constant seawater temperatures (13, 15, 17, and 19 °C) ranged from 11.1 to 12.1 mm and the mean starting total weight for each of the four groups was 0.2 to 0.3 g. Following 175 days of culture, mean shell growth was greatest at 19 °C and least at 13 °C (Fig. 7). The mean final size for these temperatures was 29.4 and 24.4 mm; a mean net increase of 17.9 and 12.3 mm, respectively. Mean weight gains were also greatest in the group cultured at 19 °C and least in the group cultured at 13 °C (Fig. 8) (Lapota 1982). The mean final weights were 3.5 and 2.1 g, a mean net weight gain of 3.3 and 1.8 g, respectively. Mean shell growth in the group cultured at 17 °C was greater than the group cultured at 15 °C (Fig. 7). The mean

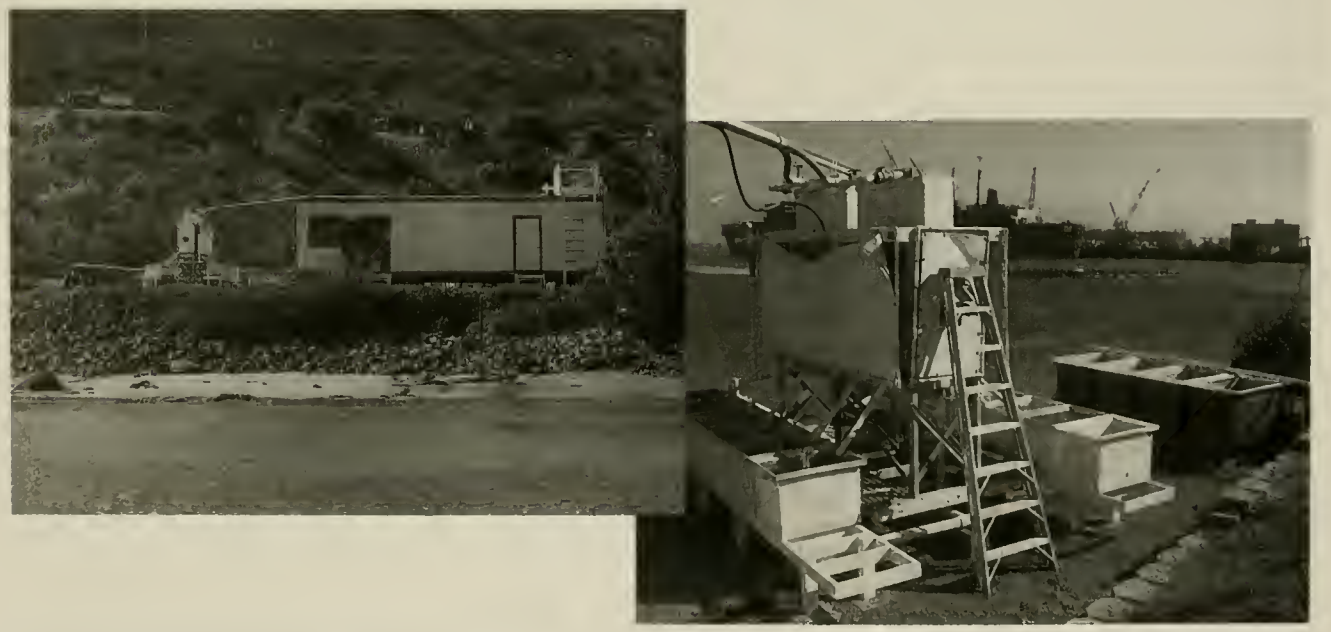

Figure 5. Photographs of the Biological Effects Program test station along San Diego Bay. The right photograph shows the POMFRET used to culture red and green abalones.

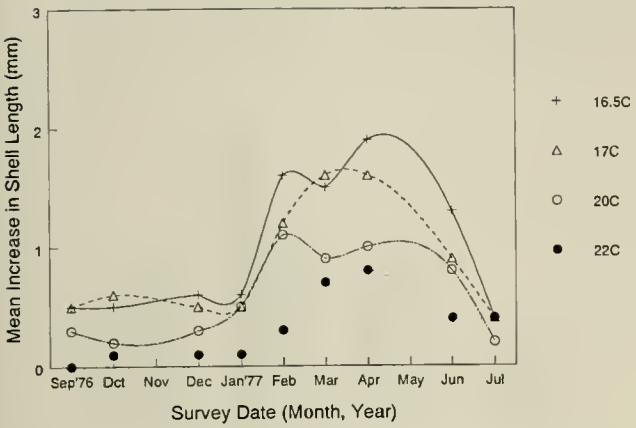

Figure 6. Mean increase in shell length of four groups of 17 hatchery-reared *H. rufescens* cultured from September 1976 through July 1977 at four constant temperatures at SPAWAR Systems Center. Measurements were taken every 30–35 days.

final size was 28.6 and 26.5 mm; a mean net increase of 17.5 and 15.1 mm, respectively. Similarly, the mean final weights of these groups were 3.0 g at 17 °C and 2.4 g at 15 °C; a mean net increase of 2.8 and 2.2 g.

Shell growth rate maximums were observed between January 30 and March 6, 1978, for the abalones cultured at 13, 15, and 17 °C (Fig. 7). Maximum shell growth rates at 19 °C were evident prior to January 30. Shell growth appears constant; however, the observed maximum rates of shell deposition add some nonlinearity to the observed growth. Shell growth rates at all temperatures decreased by May 15, 1978.

Growth of H. rufescens *on* Macrocystis *and* Egregia Kelp *Diets (HSWRI)*

Mean shell growth was fastest in both groups cultured at 19 °C and slowest at 13 °C (Fig. 9). The mean increase in shell length at 19 °C for the *Macrocystis*-fed and *Egregia*-fed groups were 3.2 and 3.5 mm/month respectively, and at 13 °C were 2.2 and 2.6 mm/month, respectively. Growth of red abalone at 15 °C fed *Egregia* was significantly greater than growth of those fed a diet of *Macrocystis*; however, there were no significant differences in rates of growth at the other culture temperatures.

There was a significant difference in the growth of red abalones

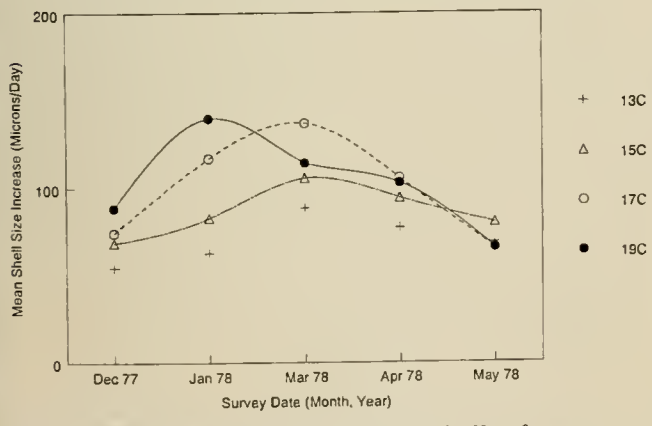

Figure 7. Mean seasonal rates of shell growth in *H. rufescens* versus seawater temperature. Growth period was 175 days.

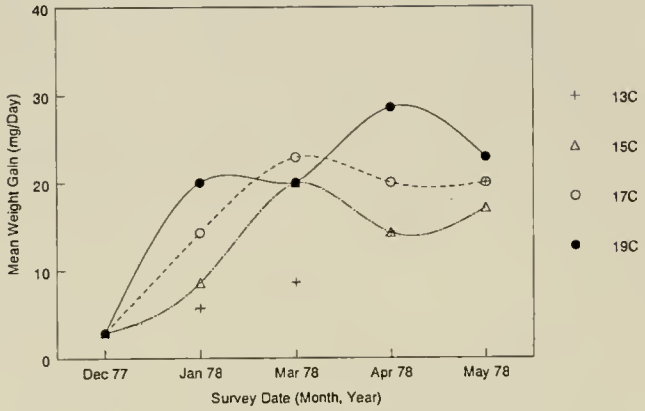

Figure 8. Mean seasonal rates of weight gain in *H. rufescens* versus seawater temperature. Growth period was 175 days.

fed *Macrocystis* at the four culture temperatures. Growth at the two higher temperatures was significantly greater than growth at the two lower temperatures of 13 and 15 °C. Also, there was a significant difference in the growth of red abalones fed *Egregia* at 13 °C when compared with growth of red abalones at the higher culture temperatures (Fig. 9).

Mean weight gains were greatest in the groups cultured at 19 °C and smallest in the groups cultured at 13 °C (Fig. 10). The average monthly growth for the *Macrocystis*-fed and *Egregia*-fed groups cultured at 19 °C were 0.6 and 0.7 g, while groups cultured at 13 °C exhibited weight gains of 0.3 and 0.4 g, respectively.

Growth of H. fulgens*—Constant Seawater Temperatures (HSWRI)*

The highest rate of growth was achieved at 21 °C, with a mean increase in shell length of 5 mm/mo (Fig. 11). Slowest growth was observed at 17 °C, with a mean increase in shell length of 1.7 mm/mo. Similarly, greatest mean weight gain (3.7 g/mo) was exhibited by the group cultured at 21 °C; lowest mean weight gain occurred at 17 °C (0.7 g/mo). There was a significant difference in growth rates of green abalones cultured at the four temperatures ($F = 36.81$). A series of t-tests showed that growth at 21 °C was significantly greater than growth at all other test temperatures ($p < .05$). The growth of green abalones at 21 °C was significantly greater than the red abalones at its optimal growth temperature of 19 °C.

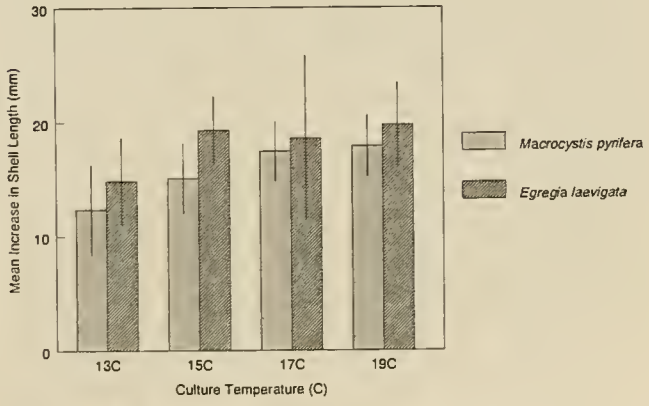

Figure 9. Mean increase and standard deviation in shell length of hatchery-reared *H. rufescens* fed two different diets versus constant temperatures. Growth period was 175 days.

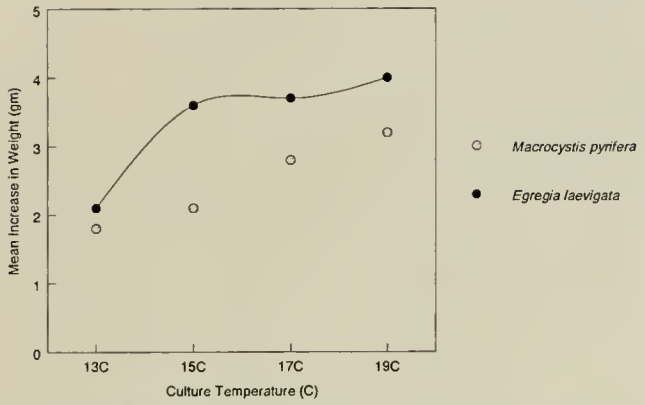

Figure 10. Mean increase in weight of hatchery-reared *H. rufescens* fed two different diets versus constant temperatures. Growth period was for 175 days.

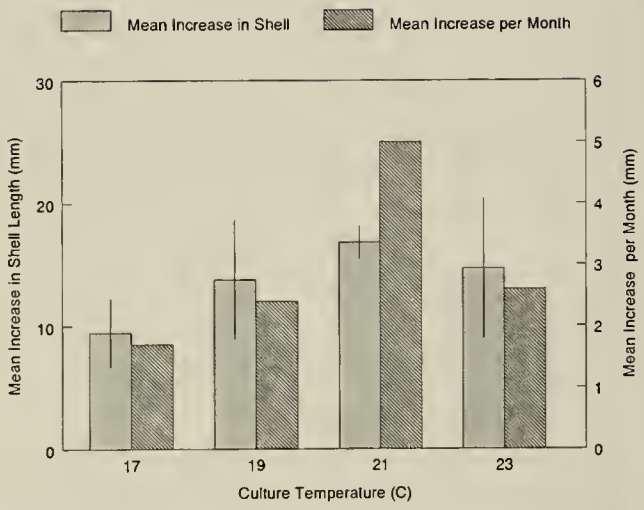

Figure 11. Mean shell length (mm) and standard deviations of four groups of 13 wild-caught *H. fulgens* cultured for 175 days at four temperatures. Measurements were taken at 35-day intervals.

Growth of H. fulgens*—Ambient Seawater Temperatures (SPAWAR)*

Abalones monitored over a 16-month period for growth increased in size, from an initial mean of 21.58 mm to 50.57 mm. Seawater temperatures throughout the year typically ranged from 14 °C in the winter months to greater than 25 °C in late summer. Growth was greater (104 μm/day) at this time (between August 10 and October 10, 1997) than during the cooler winter months (January 25–April 10, 1998) (6–25 μm/day) (Fig. 12). Weight gain in the green abalones was most pronounced from April 10 to November 4, 1998. Mean weights increased from 5 to 16 g in this 7-month period.

DISCUSSION

Shell growth and weight gain of juvenile red and green abalones appear to be temperature dependent. When constant, elevated temperatures were maintained, growth rates of red and green abalones increased substantially.

The results indicated that more rapid growth in juvenile red abalone was obtained at 19 °C, with an average daily shell growth of 0.1 mm/day. Maximum sustained growth in juvenile green abalone displayed an average daily shell growth of 0.16 mm/day at a constant culture temperature of 21 °C, similar to growth rates observed in green abalone during the summer months of 1997–1998 in Point Loma. Extrapolations of these laboratory growth rates for an entire year predict growth of 36 mm/y for red abalone and 60 mm/y of growth for green abalone. However, since ocean temperatures are not static along the coast and may have a range as much as 10–12 °C during the year along southern California, growth predictions based on laboratory-cultured juveniles may vary considerably from those observed in wild populations.

Nutritional aspects may be as important as temperature effects on growth rates in wild populations. Previous studies have observed that *Egregia* and mixed algal diets are a superior food source for juvenile abalones (Leighton 1976). However, growth rates of red abalones fed *Egregia* increased only minimally in this study (Lapota 1978). Some nutritional components of *Macrocystis*, such as protein and carbohydrate levels, vary considerably throughout the year. Additionally, C:N ratios in *Macrocystis* vary seasonally from 17:1 in the winter months to 40:1 in the summer months (Jackson 1977). Nutrient concentrations in the vicinity of kelp beds also vary with season. Below 4–5 m, nitrate concentrations are greatest during winter–spring (upwelling) and lowest during the summer.

Artificial diets containing crude protein levels (white fish meal) in excess of 30% produced increased growth in *H. discus* when compared to other artificial diets with lower crude protein levels (20–30%) (Ogino and Kato 1964). Growth rates declined when the diet contained less than 15% crude protein. Increased growth rates exhibited by juvenile red abalones in our studies from January through April 1977 may indicate changing nutrient ratios or protein levels in the kelp, which are otherwise limiting during nonupwelling months. It is probable that the nutritional quality of the kelp limits growth in the abalone even when optimal growth temperatures are present.

Past Reseeding Efforts

The Experimental Abalone Enhancement Program in California began in 1978 with four approaches to be tested for meeting the goals of the program (Tegner et al. 1981). The Department of Fish

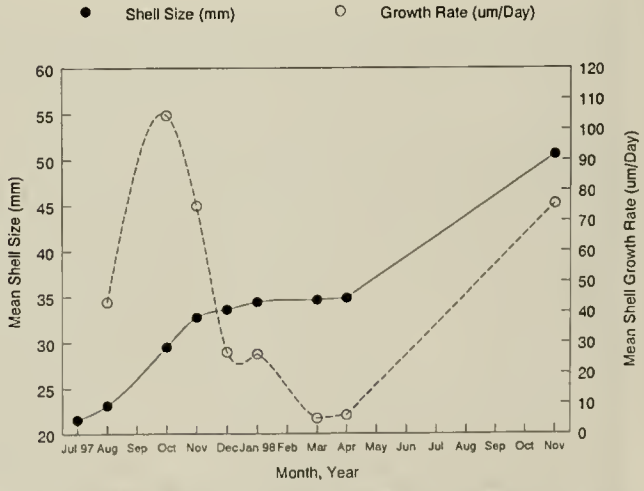

Figure 12. Mean shell length (mm) increase and growth rates of hatchery-reared *H. fulgens* cultured at ambient seawater temperatures for 16 months at SPAWAR Systems Center, Point Loma.

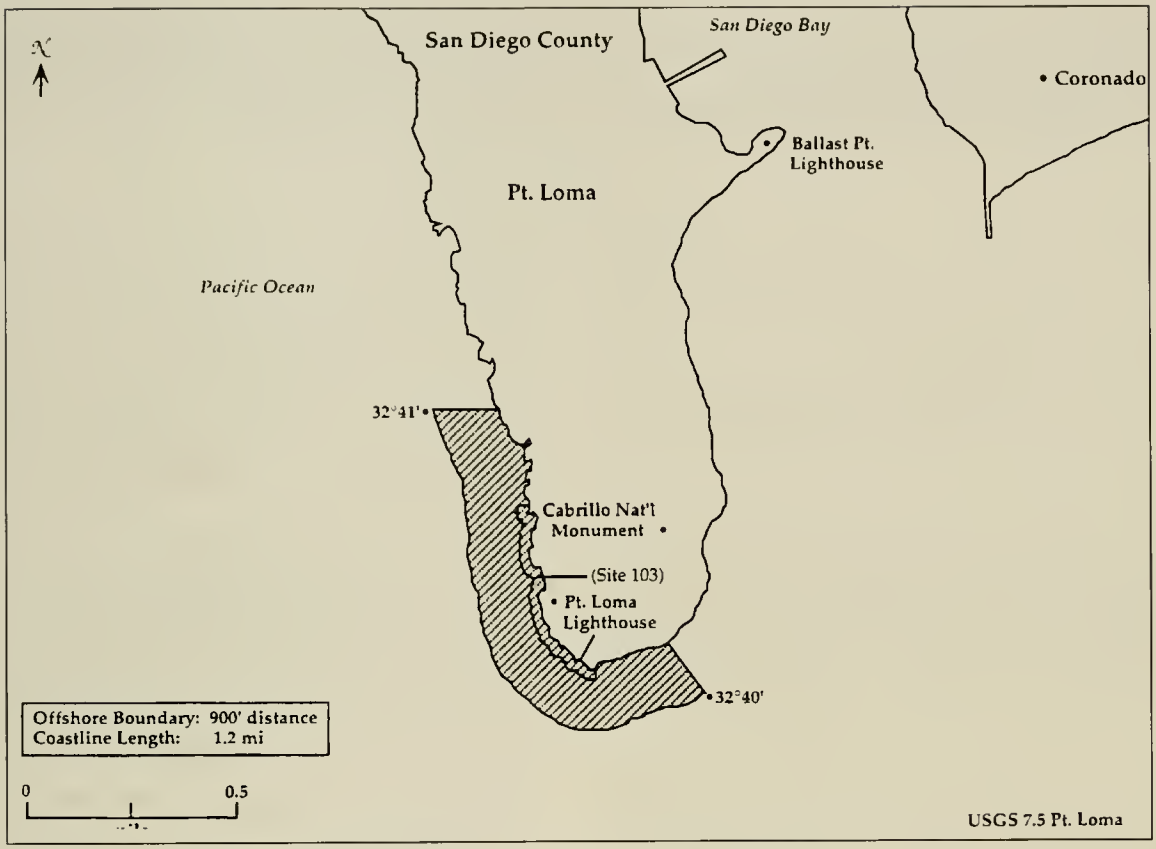

Figure 13. Proposed abalone seed planting site off Cabrillo National Monument, Point Loma, San Diego, California.

and Game closed the mainland coast between Palos Verdes and Dana Point for 5 y to all sport and commercial diving activities. This management technique was initiated to study the effects of abalone hatchery seeding activities and recruitment into the closed area. The other approaches used in the closed area were designed to observe the effects of habitat improvements and modifications on the settlement of endemic and seeded abalones, and to study the effect of transplantation of adult abalones as brood stock into the closed area.

From several large-scale seed plantings of red and green abalones conducted in various coastal sites of southern California, several important facts have emerged. Poor seed survival in transplants may be attributed to stress from transport and handling. Small abalone are cryptic and mobile, making survival assessment difficult. Some of the earlier experimental seeding activities in November 1979 in Santa Barbara County indicated that the habitat chosen for these plantings is critical to the survival of the planted seed. Approximately 9900 hatchery-raised red abalone seed, averaging 31 mm in length, were planted in a boulder habitat. Four months of surveys following the planting indicated that seeding did not increase juvenile abundance in the study area (Tegner et al. 1981). Only two hatchery-raised abalone were found. Predation of the seed by crabs, lobsters, and octopuses was observed within 2 days of the planting. Four months after another planting (mid May 1980) of 8900 red abalone seed in the same area, the experimental site was found to contain significantly more juveniles. Juvenile density changed from 0.33 per 10 m^2 to 3.88 per 10 m^2 (Tegner et al. 1981). Of the 8900 planted seed, an absolute increase of juvenile abalones in the study after 4 months was estimated between 600 and 800 abalones. Apparently, only 9% of the seed could be accounted for within the study area. Smaller hatchery seed (20 mm in length) appeared to move out of the study area at significantly higher rates than naturally occurring seed (Tegner et al. 1981). Later field experiments conducted off Palos Verdes in 1981 indicated poor recruitment irrespective of seed size (Tegner and Butler 1985). While the growth of seeded abalones was similar to laboratory-grown abalones, survival of the seeded abalones was only 1%. These results contrast with reseeding efforts conducted in Japan by the Abu Fishery Cooperative (Kojima 1995) and with the increased recruitment from planting larger seed according to Inoue (1976). Recapture rates for seeded abalones in the initial size range of 15–40 mm were from 12 to 51% in the 1980–85 year classes that had been fished (Kojima 1993). The survival rate of larger seeded abalones approached 70% survival for seed 70 mm and larger (Inoue 1976). Such different results from similar studies raise questions that can be addressed in future studies in Point Loma.

Present Activities

Based on these later observations, our group is currently growing green and red abalones seed to a size of approximately 70 mm before attempting to reseed areas of Point Loma in Cabrillo National Monument (Fig. 13). The National Park Service divided the monument area into three zones, based on accessibility by park visitors. Area III (southern tip of Point Loma) is relatively undisturbed by visitors to the park and is in close proximity to our laboratory. All of these areas contained abundant populations of abalones in the 1960s and 1970s, but have been completely deci-

mated by sport and commercial fisherman. Green abalone will be planted in the low intertidal zone and red abalone will be planted in the adjacent kelp beds at depths of approximately 8–12 m. Approximately 1000 green and red abalones at a mean size of 70 mm will be distributed along a series of transects. Growth and survival will be monitored at 6-mo intervals. The stocking density will be varied within the area to assess future seed recruitment. Hopefully, by eliminating or reducing human interference, we will be able to observe increased abalone recruitment from our near-term reseeding with larger abalone.

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THE DEMAND FOR OYSTER RELAYING ACTIVITIES IN LOUISIANA: 1976—1995

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ABSTRACT Louisiana usually leads the nation in the annual production of oyster meats. Production is derived from both leased water-bottoms and the public seed grounds. A sizeable amount of the water-bottoms under lease have either conditional or restricted status. To make the best economic use of leases under these two statuses, leaseholders will, at times, relay oysters from leases in conditional or restricted areas to leases in approved areas. This paper examines relaying activities in Louisiana during the period 1976–1995. Specifically, an econometric model was developed and used to examine the demand for relaying activities in the state. Results suggest that demand depends significantly on economic as well as environmental factors.

KEY WORDS: Louisiana, leases, oysters, public seed grounds, relaying

INTRODUCTION

About 70% of the U.S. population lives within 50 miles of the coast, and between 1950 and 1984 the population in coastal counties grew by more than 80% (USEPA 1989). This rapid rate of growth, in conjunction with the absolute number of people living along the coast (about 350 per square mile), has strained the fragile ecosystems that support shellfish populations (particularly molluscan). As a result, many of the traditionally productive shellfish nursery and habitat grounds have been destroyed and others have become unsuitable for human activities. Some of the specific human-induced causes of deterioration of shellfish-growth areas, as cited by the U.S. Environmental Protection Agency (USEPA 1990) include:

Industries–According to USEPA estimates, 1300 major industrial facilities discharge directly into estuarine and near-coastal waters;

Sewage treatment plants–Almost 600 municipal treatment plants discharge effluents into estuaries and near-coastal waters;

Nonpoint sources–More than one half of coastal pollution is attributable to urban and agricultural nonpoint sources;

Combined sewer overflow–Raw sewage and urban runoff are discharged into estuaries after rainstorms in urban areas.

Although the Gulf of Mexico is considered "the most healthy of our (i.e., U.S.) coastal marine environments" (Lipka et al. 1990), most, if not all, of the previously cited anthropogenically induced causes of deterioration of shellfish-growing waters are present to a greater or lesser extent in the Gulf of Mexico region and, specifically, Louisiana.

One readily available source for information on water quality in the Gulf of Mexico is the compilation of classified shellfish-growing waters published by the National Shellfish Register of Classified Estuarine Waters. Information on Gulf of Mexico estuarine water classifications for 1971 and 1995 is presented in Table 1. Approved growing waters constituted about 63% of total classified growing waters in 1971 but declined to less than 45% in 1995. Conditional growing waters increased from about 6% of the total in 1971 to more than 15% of the total in 1995. Restricted and prohibited waters equaled about 40% of the total in 1995 compared to only 32% in 1971.[1] With respect to Louisiana, 1.6 million acres of estuarine waters (both leased and non-leased) were classified as approved in 1995 while 400,000 acres were classified as conditional and almost 1 million acres were classified as restricted.

The compilation of shellfish-growing waters provides one indication of estuarine water quality; however, it is somewhat limited in scope (i.e., it tests only for elevated levels of indicator organisms). Estuarine water quality in the Gulf of Mexico has also changed over time as a result of physical changes in wetlands. In Louisiana, for example, wetlands are being lost at a rapid rate due, at least in part, to human action (see Turner and Cahoon 1988 for details). Alteration of the wetlands has resulted in salinity changes in many of the local ecosystems, impairing the productivity of natural oyster reefs. As noted by Van Sickle et al. (1976), the oyster industry in Barataria Basin, Louisiana, one of the most productive basins in the state, "is steadily being squeezed between encroaching salinity (and the accompanying predation and disease problems) from the south and pollution from the north" (p. 17). The encroaching salinity discussed by the authors is largely the result of human activities. These authors concluded that further coastal erosion will force production further inland, where higher levels of coliform exist and, at some point, areas suitable for oyster production will decline. In support of their conclusions, leased water bottom acreage in Louisiana advanced from 32,000 acres in 1950 to almost 400,000 acres currently. Despite this sharp increase

[1]In 1971 the restricted classification was not used. Waters classified today as restricted were classified as prohibited in 1971.

TABLE 1.

Gulf of Mexico shellfish estuarine waters classification trends, 1971 and 1995 (1000 acres).[a]

Classification	Year 1971	Year 1995	% Change 1971 to 1995
Approved[b]	3226 (62.9)[c]	2860 (44.2)	−11.4
Conditional	282 (5.5)	997 (15.4)	254
Restricted	0 (0.0)	1597 (24.7)	
Prohibited	1618 (31.6)	1015 (15.7)	−37.3
Total	5126 (100.0)	6469 (100.0)	26.2

[a] Does not include classifications of offshore growing areas and/or unclassified waters.

[b] Definitions of classifications: *Approved waters:* Shellfish may be harvested for direct marketing; *Conditional waters:* Shellfish-growing waters may be opened if they meet approved classification status under predictable conditions. Waters are opened when water quality standards are met and closed at other times; *Restricted waters:* Shellfish-growing waters can only be harvested if shellfish are relayed or depurated prior to marketing; *Prohibited waters:* Shellfish may not be harvested for direct marketing.

[c] Numbers in parentheses reflect percentage of the yearly total associated with corresponding classification. Sources: U.S. Department of Commerce (1997) and Bell (1978)

in leased acreage, annual oyster production in Louisiana has remained quite stable in the long run, generally in the range 10-million to 13-million pounds. This suggests that the productivity of the "average" lease has fallen substantially during the past several decades.

Louisiana usually leads the nation in the production of oyster meats. Production is derived from both privately leased grounds and public grounds, with production from the former historically accounting for about 80% of the state's total annual landings; in recent years, the share has fallen to approximately 50%. While production from the public seed grounds has historically been minor relative to production from the private leases, these grounds serve two important purposes. They provide a source of market oysters during the September–March period (the months during which the public grounds are generally open to harvesting activities). Second, the public seed grounds provide a source of seed oyster (i.e., less than three-inch oysters), which leaseholders can transplant to their private leases and harvest at a later time when public seed grounds are closed.

Acreage leased in conditional or restricted waters can also serve a purpose to the lease-based oyster operations, specifically oysters can be moved from conditional waters to approved waters and can be harvested when having met approved classification status. The purpose is that of using the leases for relaying activities. The U.S. Food and Drug Administration (1995) definition of relaying can be summarized as the transfer of shellfish from restricted areas, conditionally restricted areas in the open status, or conditionally approved areas in the closed status to approved or conditionally approved areas in open status for the reduction of pathogens as measured by the coliform indicator group or poisonous or deleterious substances that may be present, by using the ambient environment as a treatment status. Because all of the closure statuses in Louisiana are based on fecal coliform level criteria, the definition essentially refers to the transfer of moderately polluted oysters to open areas for natural depuration.

The process of relaying can provide both the oyster harvester and the environment with a resource restoration process. Many oyster restoration processes are basically the introduction into the environment of some sort of material for cultch attachment. The oyster larvae attach and establishment of an oyster reef community begins; in some areas of the country it takes several years for the first generation inhabitants to establish themselves. The process of relaying can be a shortcut to this process as relaying can provide both the attachment material, which in this case is the oyster shell, and a living community of oysters and fauna associated with an oyster reef environment. The area from which the relaying occurred will continue its existence and the area to which the relaying takes place will obtain, for all practical purposes, a complete restored oyster reef community.

Although potentially beneficial, the process of relaying is labor and capital intensive. Hence, it is conducted only when economic and environmental conditions indicate a favorable return on investment. The purpose of this paper is to examine the demand for relaying activities in relation to economic and environmental conditions faced by the industry during the period 1976–1995. While the study is specific to Louisiana, the results should be applicable to other states with lease-based oyster or other shellfish operations.

To achieve the purpose of this paper, a brief description of relaying activities in Louisiana is presented in the next section of the paper. Attention is then given to theoretical considerations and the development of a conceptual model that is used to determine the factors influencing the demand for relaying activities. Finally, empirical results are presented, along with a brief discussion of relevant findings.

DESCRIPTION OF LOUISIANA'S RELAYING ACTIVITIES

Relaying oysters in Louisiana requires a permit from the Louisiana Department of Health and Hospitals. The cost of the permit is nominal, approximately $50. However, the applicant is also required to post a $5000 security bond, which is returned if no violations are detected during the relaying process. In general, the permits are valid for a 2-week period.

Two types of relaying activities are practiced in Louisiana. The first is generally referred to as an experimental or controlled relay. Relays of this type are conducted infrequently (Fig. 1). The state

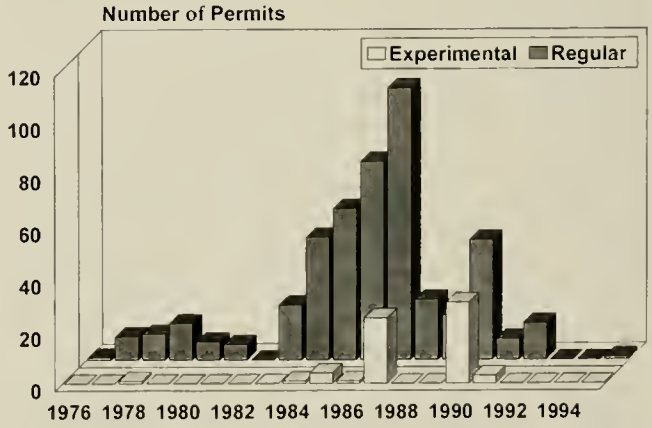

Figure 1. Annual number of experimental and regular permits issued for relaying: 1976–1995.

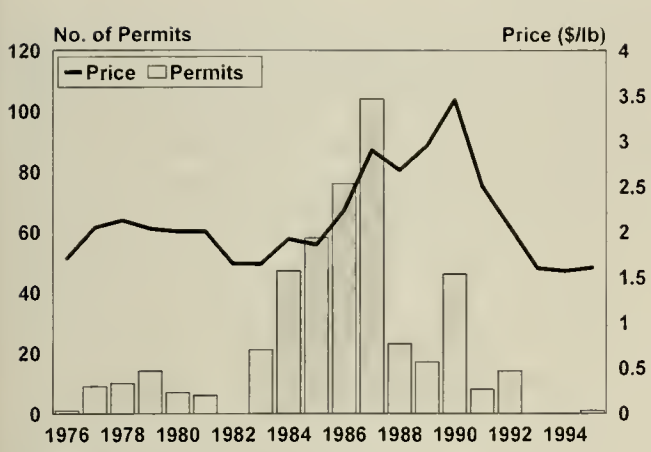

Figure 2. Annual number of regular permits issued and deflated Louisiana dockside oyster price: 1976–1995.

permits "controlled" relaying for a limited duration from polluted water bottoms in the public seed grounds or other areas not under lease. In 1990, both the public seed grounds in Vermillion Bay and the southeast corner of Lake Pontchartrain (which is neither a designated public seed ground area nor open for leasing activities) were opened for controlled relaying. In 1985, the state-owned reef in Bay Junop (that area of the reef in polluted waters) was opened for a limited amount of time for controlled relaying, and in 1987 the state-owned reef in Vermillion Bay was opened.

The second type of relaying activity is simply referred to as the regular relay. This entails the movement of oysters from leases in conditional or restricted waters to leases in approved waters.[2] These regular relays are the primary focus of this paper. As indicated in Figure 1, permits issued for regular relaying activities were consistently less than 40 per year from 1976 until the mid 1980s. Then the number of permits issued advanced rapidly, peaking at over 100 in 1987. Beginning in 1988, the number declined sharply, though a moderate increase was observed again in 1990.[3]

While considerable detail is given in following sections of the paper as to the reasons for the observed annual variation in number of regular permits issued, some general observations are presented here. First, the deflated price of the harvested product (i.e., the current price adjusted for inflationary effects) advanced rapidly during the 1985–1990 period but declined sharply in 1991, and has remained well below that observed during the mid 1980s to 1990 period (see Fig. 2). One would hypothesize, *ceteris paribus*, that increases in the deflated price of the harvested product would result in increased relaying activities.

Even though the deflated price increased through 1990, a sharp decline in the number of regular permits issued began in 1988. The Louisiana Department of Health and Hospitals initiated a requirement in 1988 that a security agent be onboard any vessel relaying oysters from polluted to approved waters. Given that this requirement would add expenses to relaying operations, one would expect

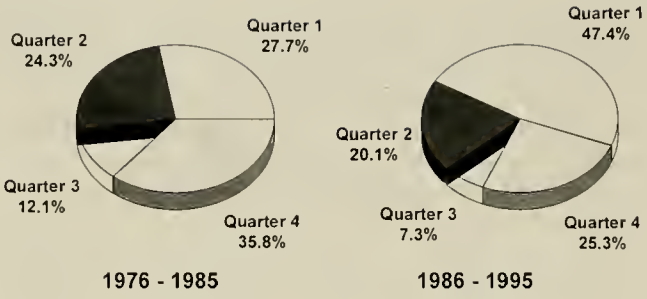

Figure 3. Quarterly distribution of issued relaying permits: 1976–1995.

that it would result in a reduced number of permit applications, *ceteris paribus*. A cursory examination of the data suggests this is the case.

The quarterly distribution of relaying permits is presented in Figure 3 for two time periods: 1976–1985 and 1986–1995. As indicated, the majority of relaying activities occurred during the first (January through March) and fourth (October through December) quarters of the year. The amount of relaying activities in the first quarter grew during the later time period. For the 1976–1985 period only about 28% of relaying activities occurred during the first quarter, but by the 1986–1995 period the percentage had increased to almost 50%.

THEORETICAL CONSIDERATIONS AND CONCEPTUAL MODEL

Theoretical Considerations

Oyster leases are an asset used in the production of market oysters. As such, an oyster lease is no different than agricultural property where the land is an asset used in the production of a crop. The implied goal of the farmer is to maximize the discounted stream of returns generated from employment of his property (owned or leased) over an infinite time horizon, or:

$$\max(NPV) = \Sigma(P_t * Q_t(q) - C_t(Q))/(1 + r)^t \qquad (1)$$

where NPV = net present value of returns from oyster farming activities; P_t = the output price of the harvested lease-based oyster product in time period t; Q_t = the quantity of oyster output from lease-based activities in time period t which in turn is a function of the quality (q) of the asset; C_t = cost of production in time period t, which is a function of output $Q_t(q)$; r = discount factor.

The net present value of returns (the discounted profits) are, as indicated, related to the output price (P_t); the output quantity (Q_t), which is related to the quality of the asset (q); the costs of production (C_t); and the discount factor (r). As specified, an increase in the output price or quantity will result in an increase in the net present value of returns. Conversely, an increase in costs for a given level of production (due to an increase in input costs) or an increase in the discount factor will result in a decrease in the net present value of returns.[4]

In general, demand for relaying activities can be considered as a derived demand for a factor of production (see Layard and Walters 1978 for details). As such, demand for relaying activities will be directly related to the ability of these relaying activities to enhance the net present value of returns from oyster farming ac-

[2]Conceivably, this type of relaying could also entail the relaying of oysters from leases in conditional or restricted waters to other leases in conditional or restricted waters. One would expect minimal, if any, activities of this nature because it would then require additional relaying activities before the oysters could be marketed.

[3]In some instances, continuations for use of an issued permit were requested and approved. These continuations are not included in the analysis.

[4]An infinite time horizon is assumed for discussion purposes. The analysis could be changed, without loss of generality, to allow for the sale of property after a fixed number of time periods.

tivities. The implications of this are multifaceted. First, it implies that the demand for relaying activities will increase (decrease) in relation to an increase (decrease) in the output price (P_t), *ceteris paribus*. Second, it implies that the demand for relaying will be positive only to the extent that it will achieve a short-run or possibly long-run increase in the output from lease-based activities (Q_t). A short-run increase in output may be achieved if the relayed oysters are removed shortly after being placed on the approved lease(s). To the extent, however, that relaying activities provide attachment material (i.e., the oyster shell and the living community of oysters and the associated fauna of an oyster reef environment), these activities can enhance the long-run productivity of a given lease.

The quantity of oyster output from lease-based activities (Q_t), as specified in Eq. (1), is a function of quality. Environmental factors, such as the salinity regime, can affect annual or even long-run quality of a lease. The derived demand for relaying activities as a function of lease quality is, to a large extent, unknown. For example, low potential production on approved acreage in a given year due to an unsuitable salinity regime may also indicate that potential productivity of leased acreage in conditional or restricted waters is also low. In this scenario, there would be little benefits from relaying as there would be little product to relay.

A third implication, based on the calculation of net present values as presented in Eq. (1), is that an increase in relaying costs will result in a reduction in the derived demand for relaying activities, *ceteris paribus*. Specifically, an increase in relaying costs results in an increase in overall harvesting cost, denoted as $C_t(Q)$. This is intuitive in that as relaying costs increase, it becomes less likely that the lease holder will achieve a positive return on his investment from relaying activities. While one generally thinks of costs in terms of inputs to the production process (e.g., fuel, crew, repairs, etc.), there are other costs to be considered. One of the most relevant is that of opportunity costs which, from an economic perspective, refers to the lost value in not pursuing the next best alternative. One alternative to relaying is transplanting from public seed grounds. If the availability of oysters (seed and market) on the public grounds is high, the need to relay from polluted to approved leases is diminished. As such, the demand for relaying, in theory, should be related inversely to availability on the public seed grounds, *ceteris paribus*.

Finally, the issue of overall market supply needs to be considered. After controlling for price and environmental factors, high volume sales may suggest readily available markets as opposed to limited markets whereby quotas may be imposed by individual dealers on the fishermen. The high volume sales associated with available markets may necessitate the need for alternative supply sources by the leaseholders. If the leaseholders are not able to secure the needed supply from their leases in approved waters or from the public seed grounds, they may turn to relaying as one method to secure the additional supply. This is of particular relevance when environmental conditions limit availability on leases in approved waters or on the public seed grounds.

Conceptual Model

Based on the above discussion, demand for oyster relaying activities in Louisiana is specified as follows:

$$\text{pera}_t = b_0 + b_1 * \text{price}_t + b_2 * \text{cost}_t + b_3 * \text{acres}_t + b_4 * \text{avpub}_{t-1} + b_5 * \text{avpri}_t + b_6 * \text{ppub}_t + b_7 * \text{ppri}_t + u_t \quad (2)$$

where pera_t = permits issued per 1,000 acres of leased water bottom in year t; price_t = deflated Louisiana dockside oyster price in year t (in \$/lb of meat); cost_t = discrete variable indicating whether security agent is required (equal to 0 before 1988 and 1 thereafter); acres_t = acres of water bottoms leased by the state for the cultivation of oysters in year t (in thousands); avpub_{t-1} = indicator of market oyster availability on public oyster grounds in year t − 1; avpri_t = indicator of market oyster availability on leased grounds in year t; ppub_t = production of oyster meats from public grounds in year t (million pounds); ppri_t = production of oyster meats from private grounds in year t (million pounds); b_0, b_1, . . . b_7 = parameters to be estimated; u_t = error term.

As indicated, the endogenous varible, permits issued in year t, is specified on a per acre leased basis (permits issued divided by acres of water-bottoms leased for the purpose of oyster cultivation). The rationale for this is that the amount of leased acreage has increased significantly during the period of analysis and if all other factors are held constant, relaying activities would increase simply because of the increased acreage. Dividing permits issued by acreage is therefore an attempt to preserve homogeneity.

The model, as given in Eq. (2), includes seven exogenous variables. The rationale for including the deflated price (price_t) has been established. Given that the demand for relaying activities is hypothesized to be positively (negatively) related to an increase (decrease) in price, the sign associated with b_1 is anticipated to be positive ($\partial(\text{pera}_t)/\partial(\text{price}_t) > 0$).

Costs, as discussed above, are also considered to influence the demand for relaying activities. Although a time-series database pertaining to harvesting and relaying costs does not exist, one cost factor in particular is hypothesized to influence the demand for relaying activities. Specifically, beginning in 1988 a requirement was enacted that a security agent be onboard the vessel when relaying activities are occurring. This requirement is thought to increase overall relaying costs significantly. To account for this increased cost, a discrete variable (cost_t) equal to 0 before 1988 and 1 thereafter was included in the model. Given that the demand for relaying activities is hypothesized to be related negatively to increased costs, the sign associated with b_2 is anticipated to be negative.

Acreage under lease (acres_t) is included in the model in an attempt to account for the long-run change in quality of the average oyster lease. As previously noted, while the amount of leased water-bottoms has increased substantially during the period of analysis, the overall long-run oyster production has remained stable, indicating a substantial decline in the average production per acre. This reduction in per acre productivity is hypothesized to be due, in part, to less desirable water bottoms being leased over time. Specifically, one would expect the more suitable oyster growing water bottoms to be leased initially. Remaining water bottoms subsequently leased are therefore of lower quality on average.[5] Relaying of oysters shell and the living community of oysters and associated fauna of an oyster reef community from leases in conditional or restricted waters to leases in approved waters is one method to enhance the long-run productivity of the leases in approved waters. Hence, one would anticipate that

[5]While this is the case on average, there are certainly exceptions. Due to wetlands erosion and subsidence, for example, many historically productive areas are now likely to be of little value in terms of their ability to produce significant quantities of oysters.

$\partial(pera_t)/\partial(acres_t) > 0$, suggesting that the sign associated with b_3 is positive.

Indicators of oyster availability on public grounds in year t − 1 ($avpub_{t-1}$) and privately leased acreage ($avpri_t$) in year t were included as explanatory vehicles in the analysis. The indicator of availability on the public seed grounds included seed and market oysters and was based on square-meter samples collected by the Louisiana Department of Wildlife and Fisheries in August each year. It is stated in terms of total estimated sacks available on the public seed grounds (in millions).[6] As estimated availability of seed and market oyster on public seed grounds increases, in theory, there should be less need to relay from conditional or restricted water-bottoms to leases in approved waters.[7] Hence, it is hypothesized that the sign associated with the coefficient b_4 is negative. The indicator of oyster availability on leased grounds was the estimated amount of wetland acreage throughout coastal Louisiana that had a salinity regime ≥10 ppt in April of each year; expressed in millions of acres.[8]

Finally, variables representing annual production from public grounds ($ppub_t$) and private grounds ($ppri_t$) were included in the analysis. As previously discussed, high levels of sales, after controlling for price and environmental factors, may suggest increased marketing opportunities. High volume sales, in turn, may indicate a need to secure additional sources of product from nontraditional sources. One of these sources is that of relaying oysters from polluted to approved waters. Hence, the expected signs associated with both b_6 and b_7 are hypothesized to be positive, that is, $\partial(pera_t)/\partial(ppub_t)$ and $\partial(pera_t)/\partial(ppri_t) > 0$.

Statistical Considerations

The model developed in the previous section can be expressed in matrix form as follows:

$$y_t = X_t b + U_t \quad \text{if } X_t b + U_t > 0 \qquad (3)$$
$$y_t = 0 \quad \text{if } X_t b + U_t \leq 0 \quad t = 1, 2, \ldots, N$$

where y_t = dependent variable, X_t = vector of independent variables, U_t = error term assumed iid $N(0,\sigma^2)$.

The model specified is referred to as the Tobit model and is used often in economic studies, particularly those of a cross-sectional nature (see Amemiya 1984 for details). Given the specification, an assumption is implicitly made that an underlying stochastic index equal to $X_t b + U_t$ is observed only when strictly positive. In other words, y_t will be positive given a value of $X_t b + U_t$ greater than zero. Otherwise, y_t will equal zero. For example, consider 2 years having identical attributes with the exception of output price ($price_t$). Furthermore, assume relaying activities were observed in the year when output price was higher, and in the year where output price was lower, no relaying activities were observed. This would imply that price in the year with the higher price had exceeded that threshold level required to relay oysters (i.e., $X_t + U_t > 0$), while price in the other year was below the threshold that would be required to relay oysters (i.e., $X_t + U_t < 0$). Factors such as those specified in Eq. (2) (i.e., those in the matrix X_t) likely influence relaying and thus the Tobit model is appropriate for the current analysis.

TABLE 2.

Summary statistics for Tobit analysis of Louisiana oyster relaying activities, 1977–1995.

Variable	Parameter estimates β_i	Asymptotic t-ratio	Regression Coefficient $\partial E(pera_t)/\partial X_i$	Elasticity of $E(pera_t)$
$price_t(\beta_1)$	4.971	4.149	0.1164	3.156
$cost_t(\beta_2)$	−14.098	−5.204	−0.3301	—
$acres_t(\beta_3)$	0.097	4.738	0.0023	8.235
$avpub_{t-1}(\beta_4)$	−0.814	−2.429	−0.0191	−0.194
$avpri_t(\beta_5)$	0.487	0.906	0.0114	0.215
$ppub_t(\beta_6)$	0.753	2.467	0.0176	0.613
$ppri_t(\beta_7)$	0.657	3.134	0.0154	1.607
constant(β_0)	−36.725	−5.316	−0.8600	—

As shown by Greene (1981), ordinary least squares (OLS) estimates of Eq. (3) are both biased and inconsistent due to nonnormality of the error terms. Thus, some estimation procedure other than that of OLS must be used if unbiased or consistent parameter estimates are to be obtained. Because several different estimation procedures have been developed and used and should in all cases provide the same parameter estimates assuming a unique maximum, the different estimation approaches are not considered here. For purposes of analysis, the software package SHAZAM version 7.0 (White 1993) was employed.

The unconditional expected value of the dependent variable in Eq. (3) was shown by Tobin (1958) to equal:

$$E(y) = X\beta F(Z) + \sigma f(Z) \qquad (4)$$

where: $Z = X\beta/\sigma$, $f(Z)$ = unit normal density function, $F(Z)$ = cumulative normal distribution function.

The unconditional expected value of the dependent variable, $E(y)$, represents the expected value of the dependent variable associated with all observations. The change in the unconditional expected value of the dependent variable with respect to a change in any exogenous variable can be expressed as:

$$\partial E(y)/\partial X_i = F(Z)\beta_i \qquad (5)$$

This expression is equivalent to the parameter estimates generally associated with OLS estimation.

EMPIRICAL RESULTS AND DISCUSSION

Results of the Tobit analysis related to Louisiana relaying activities during the 1977–1995 period are presented in Table 2.[9] The first column lists the variables used in the analysis. The Tobit parameters associated with each of the exogenous variables are given in the second column. The asymptotic t-values associated with the parameter estimates are presented in the third column of the table. The unconditional or total expected change in the dependent variable ($pera_t$) due to a change in the specified exogenous variables is given in the fourth column. Finally, the unconditional elasticities, which measure the expected change in the dependent variable ($pera_t$) with respect to a 1% change in any of the inde-

[6]Only data for the public seed grounds east of the Mississippi River (i.e., Breton Sound seed grounds) were used in the analysis.

[7]The indicator of availability on public seed grounds was lagged by one period in the analysis. This reflects the fact that the survey taken to estimate availability is conducted in August of each year and harvesting activities for either seed or sack production is permitted from about September through the following March. Hence, it is the estimate of availability from time period t − 1 that will influence relaying activities in time period t.

[8]It is generally believed that an extended salinity regime <5 ppt results in high mortality rates. The data required to construct a variable using the 5 ppt criteria was not available for the current study.

[9]Due to the inclusion of a lagged variable ($avpub_{t-1}$) in the regression model, the first year (i.e., 1976) is lost from the analysis.

pendent variables (evaluated at the means), are presented in the last column of the table.[10]

The parameter estimates associated with relaying activities appear satisfactory and reasonable based on two criteria. First, all estimates, with the exception of the indicator of oyster availability on private leases ($avpri_t$), conform to theoretical expectations with respect to parameter sign. Second, the asymptotic t-values of all parameters with the exception of $avpri_t$ are significant at the 95% confidence level.[11] Given the fact that no previous research has been conducted in this area, comparison of the magnitudes of the current parameter estimates cannot be compared with results from previous research to ascertain conformity across studies.

The results indicate that a one dollar increase in the deflated dockside price of the harvested product in year t will result in an increase of 0.116 permits issued on a per acre basis. At the mean value of leased acreage during the period 1976–1995 (285,000 acres), this translates into a demand for an additional 33 permits for every one dollar increase in the deflated price, *ceteris paribus*. Similarly, a 10% increase (decrease) in the deflated price was estimated to result in excess of a 30% increase (decrease) in the demand for permits per acre of water-bottoms leased. While derivation of an elasticity with respect to $cost_t$ is not valid due to the discrete nature of the variable, the results suggest that the requirement of the Louisiana Department of Health and Hospitals that a security agent be onboard vessel when relaying reduced significantly the demand for relaying activities.

Increases (decreases) in the estimated availability of seed and sack oysters on the public grounds in year t − 1 were found to reduce (increase) significantly the demand for relaying activities in the current year. Overall, a 10% increase in $avpub_{t-1}$ was found to result in a decrease in $pera_t$ of approximately 2%. At the mean number of acres leased (258,000), a 1 million sack increase in available supply (seed or market oysters) was estimated to result in an overall reduction of five permits issued for relaying activities, *ceteris paribus*.

The indicator of availability on private leases ($avpri_t$) was not found to significantly influence the demand for relaying activities. There are at least two possible explanations for this finding. First, the indicator (i.e., coastal acreage with a salinity regime in excess of 10 ppt) may not fully reflect annual variation in productivity of leases due to annual environmental changes. Second, the environmental factors that determine productivity on leases in approved waters may, in similar fashion, determine productivity of leases in polluted waters. Hence, if availability is low on leases in approved waters, it will also be low on leases in polluted waters and costs of relaying would, in turn, be prohibitive.

The results suggest that a 1 million pound increase in market oyster production from public seed grounds was found to result in an increase of 0.018 permits per acre leased. This translates to an increase demand for permits equal to 4.5 when evaluated at the mean number of acres leased during the period of analysis (258,000). With respect to production from leased acreage, the increase demand for permits at the mean amount of acreage leased equaled 4.0. This suggests that increases in production from either the public grounds or the private grounds have approximately the same impact on the demand for permits to relay. When examined on the basis of elasticities, the analysis suggests that a 10% increase in the market production from public grounds can be expected to result in approximately a 6.1% increase in the demand for permits while a 10% increase in production from the private grounds results in a 16% increase in demand for relaying activities.

Overall, the results support the hypothesis that economic and environmental factors largely determine the demand for relaying activities. While controlling environmental factors that influence relaying activities is outside the control of management agencies, the development of options to control economic factors, particularly costs, may be feasible. Controlling costs will, under optimal conditions, encourage increased relaying activities. Because the process of oyster relaying can be a shortcut to the establishment of oyster reef communities when compared to other restoration processes, further examination of the issue is warranted.

[10]An elasticity with respect to the variable $cost_t$ was not derived because of its discrete nature.

[11]While possibly biased due to the limit observations in the analysis, ordinary least square tests also suggested that the model performed adequately. Specifically, the adjusted R^2 equaled 0.93 and, based on the Durbin Watsin test statistic, there was no indication of serial correlation.

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COMMUNITY INVOLVEMENT IN PROJECTS TO REDUCE NONPOINT SOURCE POLLUTION

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ABSTRACT Baynes Sound, located on the east coast of Vancouver Island in western Canada, is one of the prime shellfish culture areas of British Columbia. In 1994 the Baynes Sound Stewardship Action Group, a multistakeholder group, was formed to address and to remediate nonpoint sources of bacteriological pollution threatening the economic and environmental health of the sound. Remediation action projects for urban stormwater, malfunctioning on-site septic systems, and agricultural runoff have successfully involved hundreds of community volunteers and dozens of financial supporters in simple and effective pollution reduction activities. The community-based "hot spots" projects have engaged and educated citizens in actions to improve water quality, in ways government authorities alone could not have done. This work demonstrates that partnerships among government, the shellfish industry, community groups, and citizens can create a powerful means for improving water quality. The projects also show that citizens are capable of creating positive environmental changes in their communities through monitoring, education, and remediation programs. Citizen volunteers can be dedicated and passionate agents of change when provided with the opportunity to participate in creating healthier communities that benefit themselves and a clean-water-dependent industry, such as shellfish culture.

KEY WORDS: nonpoint source pollution, citizen participation, bacteriological pollution, urban stormwater, agricultural runoff, malfunctioning on-site septic systems

INTRODUCTION

Involving citizens in protecting water resources is recognized as being a powerful tool for protecting water quality (Broadhurst 1996). Throughout Canada and the United States, funding agencies have increasingly recognized the importance of community-based projects and the value of environmental stewardship. This paper outlines the actions that one western Canadian community has taken to protect their local water resources from nonpoint source pollution.

Baynes Sound, located on the east coast of Vancouver Island in western Canada, is a prime shellfish culture area, producing 40% of all shell stock in the province of British Columbia (Comox Valley Economic Development Society 1998). The shellfish industry of Baynes Sound comprises 520 ha of intertidal area with 115 shellfish growing leases. This clean-water-dependent industry has a current wholesale value of $6 million (CDN) primarily in Pacific oysters, *Crassostrea gigas*, and Manila clams, *Tapes philippinarum*. The geoduck clam, *Panope abrupta*, harvest, which is still in its infancy, generated $12 million revenue in 1997 for license holders in the region (Comox Valley Economic Development Society 1998).

Water quality deterioration of Baynes Sound was first identified in 1994 by Environment Canada during their Canadian Shellfish Sanitation Program (CSSP) survey. This decline in marine water quality, attributed to nonpoint source pollution, resulted in closure of 20% of the shellfish harvesting area in 1994 and has had a direct impact on the shellfish industry.

The Baynes Sound Round Table and the Baynes Sound Stewardship Action Group (BSSAG) formed in 1994 to address and to remediate nonpoint sources of bacteriological pollution threatening the economic and environmental health of the sound. BSSAG is a multistakeholder group with representatives from four government agencies, the shellfish industry, and three community groups. The group has collectively designed pollution reduction projects for urban stormwater, malfunctioning on-site septic systems, and agricultural runoff, which have successfully involved dozens of financial supporters and hundreds of community volunteers in simple and effective pollution control activities.

The partnerships formed among government, industry, community groups, and citizens have created an action-focused group capable of garnering sufficient financial support for the monitoring, education, and remediation work. The representatives from the multistakeholder group bring their knowledge and resources to further their collective goal of clean water.

The initial inspiration and ideas for this community-based pollution reduction approach came from activities undertaken by the Washington Sea Grant Program and the Puget Sound Water Quality Authority in Puget Sound, Washington state, USA. The projects undertaken in the Comox Valley were based on their work but were redesigned to fit the needs and characteristics of the local community (Pinho 1998b).

The community projects described in this paper summarize the activities of approximately 200 local citizen volunteers involved in pollution monitoring, education, and remediation activities. These citizens have become informal teachers to their neighbors, coworkers and families, encouraging people to see that Baynes Sound belongs to all residents, who share a collective responsibility for water quality. Each of the programs aimed at reducing nonpoint source pollution included educating people about their personal actions, their impacts on water quality, and the effects of shellfish contamination. Citizens were given the "tools" to become better stewards and to educate others that water quality in the Baynes Sound is a community responsibility (Pinho 1998b).

The community-based projects were designed to address pollution from stormwater discharges, failing on-site sewage systems, and agricultural runoff. Each of the programs involved citizens in the field activities and also in decision-making for designing the programs and their future directions.

URBAN STORMWATER MONITORING AND REPAIR OF SEWAGE CROSS CONNECTIONS

The stormwater-monitoring project, managed by the Comox Valley Project Watershed Society, involved citizens in identifying

pollution sources, prioritizing them, and reporting the results to the two local governments. In 1996, forty community volunteers were trained to monitor bacteriological water quality. They monitored 60 storm drains once per month for a 6-month period, collecting a total of 381 fecal coliform samples (Pinho 1996). Of the 60 storm drains monitored, 16 drains posed a high risk to shellfish or human health (greater than 1000–5000 coliform fecal units/100 mL). As a result of this intensive data collection and sharing, the City of Courtenay and Town of Comox became aware that there were sanitary sewer-storm drain cross connections.

A sanitary sewer-storm drain cross connection occurs when a domestic sanitary sewer service is mistakenly connected to the storm drain rather than to the sanitary sewer, thus carrying untreated household domestic sewage down the storm drain. This plumbing error was completely unknown to the two municipalities prior to stormwater testing by citizen volunteers. Over 70 sanitary sewer-storm drain cross connections were repaired between 1996 and 1998, and repairs are continuing. The City of Courtenay alone has spent $110,000 (CDN) on cross connection repairs from 1996 to 1998. In 1996, the two municipalities also purchased smoke-testing equipment, an essential tool for locating the specific lines in need of cross connection repairs (Pinho 1998c).

Figure 1 illustrates the dramatic difference between fecal coliform outputs prior to and after cross connection repairs. Fecal counts from this one storm drain, which discharges directly into north Baynes Sound, now average 60 CFU/100 mL versus previous counts as high as 3 300 000 CFU/100 mL. Seven cross connections were repaired at this site in 1996 and two repairs were made in 1998 (Pinho 1996). These cross connection plumbing errors were located in a residential complex constructed in the 1950s, and this source of fecal pollution continued unnoticed for 46 years. This pollution source was discovered and acted upon only after community volunteers donated their time to water quality monitoring. During this 6-month monitoring period, citizen volunteers collectively donated 720 hours to testing storm drains.

ON-SITE SEWAGE SYSTEM CARE AND MAINTENANCE EDUCATION PROGRAMS

Sixty-five on-site septic system inspections were performed randomly throughout the Baynes Sound area in 1996 (on systems volunteered for inspection). In this crude study, undertaken with the assistance of a septic company, systems fell into one of four categories: 17% were passing and functioning well; 42% were displaying evidence of limitations; 23% were pre-failing, and 8% were absolutely failing systems (Drake 1997). The study results indicated a need for improved education, routine maintenance, and, in some cases, complete replacements of on-site septic systems in the region.

Proper septic care and maintenance education campaign workshops, called septic socials, and community septic tank pump-outs were conducted in 1996 and 1997. The Comox Valley Citizens Action for Recycling and the Environment (CARE) managed these efforts, which included printing approximately 1000 information kits for distribution (Drake 1997). Eighty community volunteers were involved in distributing the educational information kits to their neighbors throughout the region, and inviting them to a septic social and oyster barbecue at a nearby resident's home

The septic socials have proven to be very popular and entertaining workshops, and have provided training to 113 rural residents on the proper functioning, ongoing maintenance, and trouble shooting of septic systems. The workshop involves a slide presentation, a video presentation, and an actual inspection of a local septic system, by a septic pumping and maintenance company. Neighbors living in rural areas often live great distances from one another, thus making this popular event an attractive opportunity for neighborhood socializing.

An interactive full-size model of a septic system was constructed for the program to further educate and involve citizens in understanding the proper functioning of septic systems. The system is made largely of a PVC pipe frame with ripstop nylon covers illustrating the general concept of a distribution box, septic tank, and distribution pipes. The model is set up at community events and parents are encouraged to "flush" their children down the play toilet and have them crawl through the distribution box, into the septic tank, and then "flow" out one of the three distribution pipes. This has become a hugely popular and fun educational tool for approaching rural residents on this unfamiliar subject.

ADDRESSING AGRICULTURAL RUNOFF THROUGH STREAMSIDE FENCING AND VEGETATION

Between 1997 and 1998, an agricultural program aimed at pollution prevention and fish habitat protection contacted 200 farmers. This landowner contact program was managed by the Comox Valley Project Watershed Society. In 1998, hobby and commercial farmers were offered up to $700 to assist them with remediation activities, resulting in the installation of 18,882 feet of streamside fencing to limit livestock access to streams on 17 farms. In addition, 6000 trees and shrubs were planted on 26 farms, covering over 7500 feet of riparian area (Pinho 1998a). The financial incentive in the program proved to be an essential element for the success of this project; farmers were given an economic rationale for environmental protection in this agricultural valley ribboned with salmon streams that flow into shellfish-growing areas downstream.

The projects emphasized protecting streambanks and developing partnerships with farmers to assist them with stream protection projects on their properties. The trust and partnerships developed among farmers, the 88 citizen volunteers, and the nonprofit society, were also a significant accomplishment for the long-term protection of water quality and fish habitat in this agricultural valley in the Baynes Sound watershed.

The program involved the farming community in the design and delivery of the program. The project was guided by a volunteer advisory committee and farmers were visited by the program coordinator and a retired farmer. The insight and credibility gained from involving the agricultural community in the program management was an essential component for farmer participation in this project.

THE OVERALL IMPROVEMENTS IN WATER QUALITY

It is difficult to summarize quantitatively the overall effectiveness of these land-based water quality projects on marine water quality. Due to funding constraints, most of the projects, with the exception of the stormwater-monitoring program, lacked detailed spatial and temporal monitoring to assist in any proper assessment.

However, monitoring was conducted at five stations every two weeks in north Baynes Sound, Comox Harbour. The marine water quality monitoring for fecal coliform was conducted from 1995 to 1997 by trained Coast Guard Auxiliary volunteers. The results of the marine monitoring effort indicate an improving trend in bacteriological water quality. From 1995 to 1997, the frequency and magnitude of fecal coliform counts greater than 43 MPN/100 mL decreased at all five stations in Comox Harbour. However, these lower levels were also coincident with lower rainfall levels experienced in the second year of monitoring. Additional monitoring is required to provide any definitive conclusions on marine water quality improvements in the region (Pinho 1998d).

CONCLUSIONS

The clean-water-dependent shellfish industry can benefit from forming alliances with government, community groups, and citizens to reduce nonpoint sources of pollution. Community volunteers can be capable of creating tangible improvements in water quality through monitoring, education, and restoration projects. Citizens involved in these water quality projects often become informal teachers to their neighbors, coworkers, and families, encouraging people to see that the community shares a collective responsibility for water quality and for protecting clean-water-dependent industries, like shellfish culture.

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MAINTAINING THE SHELLFISH NICHE IN THE NEW MILLENNIUM: THE PACIFIC COAST SHELLFISH GROWERS APPROACH

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ABSTRACT Intense population growth, associated nonpoint source pollution, competing uses of the estuaries (e.g., pristine views, recreation) and increased regulatory scrutiny associated with the Endangered Species Act and the Sustainable Fisheries Act are jeopardizing the survival of West Coast shellfish farmers. The Pacific Coast Shellfish Growers Association (PCSGA) has recognized that to maintain a niche for shellfish amongst all these competing interests, growers need to be proactive on several fronts. PCSGA members are encouraged to get involved with local watershed planning committees, growth management planning, Soil Conservation Districts, environmental groups, and other watershed stakeholder groups, and to educate others on the value of shellfish as part of a healthy ecosystem. Shellfish farmers are also being encouraged to "walk the talk." It is no longer adequate to go to hearings, profess shellfish as the "canaries in the mineshaft," and ask everyone upstream to fix the problems. Shellfish growers are being asked to review and improve their overall environmental performance. PCSGA is embarking on an effort to develop a coastwide Environmental Code of Practice (Best Management Practices) for shellfish farming. PCSGA is also exploring the development of the Environmental Code of Practice into a programmatic Habitat Conservation Plan to provide regulatory stability under the Endangered Species Act and impending salmonid listings. The Pacific Shellfish Institute (PSI), the research arm of PCSGA, is pursuing funding for shellfish ecosystem research as a high priority. Being proactive and establishing the industry as the most responsible user group in the ecosystem will assure that shellfish farming survives into the new millennium.

KEY WORDS: Environmental management system, environmental policy, environmental code of practice, ecosystem, eelgrass

INTRODUCTION

The Pacific Coast Shellfish Growers Association (PCSGA) is the predominant shellfish industry organization of the United States West Coast. PCSGA represents oyster, Manila clam, mussel, and geoduck farmers. The Association, formed originally in 1930 for the annual procurement of Pacific oyster seed from Japan, currently deals with a variety of environmental, water quality, health, and regulatory issues coastwide.

The West Coast shellfish industry began in the late 1800s with wild harvest of the delicate native Olympia oyster, *Ostreola conchaphila*, in Washington, Oregon, and California. Harvest pressure and pollution forced a transition to hardier, more prolific Pacific oysters, *Crassostrea gigas*, beginning in 1921. While Pacific oysters grew well on the West Coast, natural reproduction was limited, requiring growers to obtain seed annually from Japan. Piggy backing in the cases of seed from Japan was the Manila clam, *Tapes philippinarum*. It adapted well to the Pacific Northwest climate, reproducing naturally in a number of bays and estuaries throughout Washington and British Columbia, Canada. It has been a focus of cultivation efforts since the 1960's. Blue mussels (*Mytilus trossulus* and *Mytilus galloprovincialis*) have also been cultured on a limited but increasing basis since the mid 1980's, mainly in Washington. Although the Pacific oyster is the predominant species cultured, a variety of other oysters (*Crassostrea virginica*, *Crassostrea sikamea*, *Ostrea edulis*, and *Ostreola conchaphila*), are cultured for the half-shell market.

Washington State is the dominant shellfish producer on the West Coast and, consequently, home to PCSGA. The relative production of cultured shellfish is represented in Figure 1. Production estimates for the various species, compiled by the Pacific Shellfish Institute (PSI), are presented in Table 1.

The past two decades have brought a number of significant regulatory and environmental issues to bear on the shellfish industry. In 1988, the PCSGA recognized it was loosing ground in a variety of arenas. Shellfish growing waters were being downgraded at an alarming rate. Population growth and shifting demographics were taking their toll both from a standpoint of pollution and competing uses displacing shellfish farms. Growers made a decision to expand from an office in Coast Oyster Company's plant in Seattle, Washington, with a 1/3 time volunteer, to a dedicated office in Olympia, the Washington State capitol, with a fulltime lobbyist-executive director. Since the expansion, the organization has been lobbying actively on environmental and regulatory issues, educating the government and the public about the benefits of shellfish farming, partnering with the environmental community, and promoting strong credible working relationships with regulatory agencies.

HUMAN IMPACTS

Socio-Political Changes

There has been tremendous population growth in the coastal areas and as a result shifts in usage in many of the rural areas traditionally farmed for shellfish. With a general decline in wild fisheries and timber-related jobs, many of the coastal areas are shifting from natural-resource-based economies to service-based and high technology economies. Loggers and fishermen are moving out and retirees and service workers are moving in. These population increases and shifts in the employment base bring a variety of environmental and sociopolitical pressures. Newcomers to the rural watersheds tend to be less accepting of the traditional working waterfront than are their predecessors. These newcomers are demanding that forests, riparian areas, beaches, and coastal and wildlife habitats be preserved and enhanced (Huppert et al. 1998). The increasing high technology, white collar work force places a greater importance on protecting natural amenities and preserving opportunities for outdoor recreational experiences. These newcomers have a higher level of environmental awareness and tend to be less accepting of unsightly aquaculture operations.

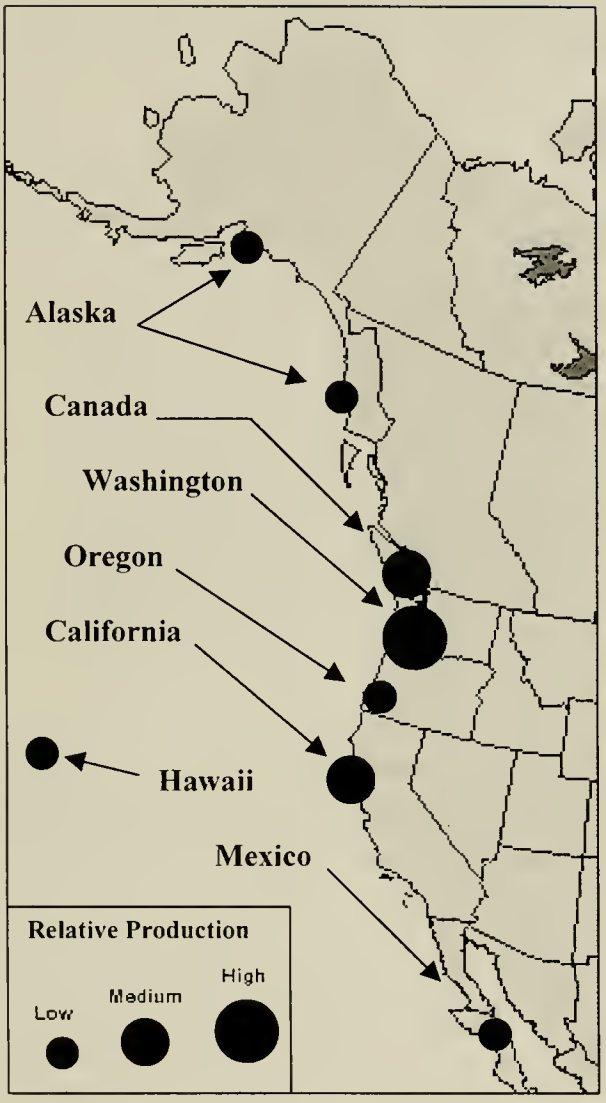

Figure 1. Relative cultured shellfish production on the West Coast of North America.

The waterfront properties adjoining shellfish beds are being developed with affluent homes. These new homes are driving up property values along the waterfront, resulting in higher property taxes. These higher taxes are impacting aquaculture operations, which are dependent on waterfront property for facilities, and are also forcing longtime residents to sell and move inland.

Increased Pollution

The increased number of people means increasing nonpoint pollution. Homes built in these rural areas typically use on-site sewage systems. If local governments do not have effective education, operation, and maintenance programs in place, the on-site systems are prone to failure, which results in shellfish growing area downgrades. Increased development also brings with it a higher percentage of impermeable surface and increased storm-water runoff. With this storm-water runoff comes untreated domestic animal waste, lawn fertilizers, pesticides, and polyaromatic hydrocarbons (PAHs).

TABLE 1.

Whole wet weight and value of production for shellfish produced on the West Coast, USA, in 1998 (PCSGA).

Species	Production (million lbs.)	Production (metric tons)	Value (million U.S. $)
Oysters (all forms)	88.6	40,200	55.0
Manila clams	7.0	3170	21.0
Mussels	1.5	680	2.5

Competing Uses

More people mean more competition among users, not only for pristine views but also for water skiing, windsurfing, personal watercraft, fishing, and pleasure boating. The increased recreational use comes with spatial conflicts and results in increased pollution from boats without sewage holding tanks, inadequate boater pump-outs for those that do, and insufficient shoreside toilet facilities.

REGULATORY IMPACTS

Endangered Species Act

The Endangered Species Act (ESA) potentially may have a very significant effect on the shellfish aquaculture industry. Declining wild fisheries, and in particular the declining salmon fishery, are drawing significant attention to habitat concerns from natural resource managers. In Washington State, both Puget Sound Chinook salmon and Hood Canal summer chum salmon were listed in March 1999 as threatened under the Endangered Species Act. Other coastal salmon runs are already listed or have listings pending. Section 9 of the ESA prohibits "take" of threatened or endangered species. A "take" not only constitutes killing the species but includes any activity that hurts or harms any aspect of the species' lifecycle, including damaging critical habitat. Critical habitat is not only habitat that is critical to the juvenile salmon, but includes habitat that is critical to the salmonid prey species (e.g., eelgrass is critical for herring to spawn on, herring is a critical salmonid prey species, therefore eelgrass is a critical habitat).

The potential impact of the ESA is related to the anadromous life cycle of salmon. Depending on the species, out-migrating juveniles can spend several days to weeks utilizing the intertidal areas. These young fish will migrate in and out with the tide, staying in shallow water to minimize their exposure to predators while feeding on abundant marine invertebrates. This same intertidal area is used for shellfish culture and, generally speaking, there is a limited understanding regarding use of the marine estuary by juvenile salmonid.

Shellfish growers conduct a variety of activities in the day-to-day operations of their farms, which could impact salmonid critical habitat during the out-migration of juveniles. Many of these activities have been practiced for decades and, in some cases, are "grandfathered" by state or federal agencies as acceptable. All activities, even those grandfathered, are now falling under a new level of regulatory scrutiny with listings of salmon as threatened and endangered species. Individuals found guilty of a "take" under ESA are subject to fines and/or prison.

Shellfish farming activities that may be coming under new or more intense review include:

Intertidal off-bottom rack and bag or longline culture for oysters. The structures used in this type of culture have the potential to impact eelgrass and other submerged aquatic vegetation (SAV) through shading effects. Depending on the site and the orientation,

the structures may cause suspended silt to settle out, modifying the bottom substrate, and thus impacting salmonid prey species. The structures, depending on their orientation, have the potential to lead juveniles following the shoreline to deeper water and greater predator exposure.

Traditional bottom culture for oysters. Bed preparation, crop management, and harvest vary from farm to farm.

Clean-up dredging. Often thick beds are hand-harvested, and dredged prior to planting to clean up the remaining oysters.

Burrowing shrimp control. Over the last 30 years, several West Coast estuaries have experienced dramatic increases in populations of burrowing shrimp (*Upogebia pugettensis* and *Callianassa californiensis*). These shrimp make oyster beds unstable, causing crops to sink and perish. Considerable effort has been expended by the industry since the early 1960s to research methods to control burrowing shrimp. In the early 1960s, the Washington Department of Fisheries (WDF), now the Washington Department of Fish and Wildlife (WDFW), working with other public agencies and the growers, found carbaryl (Sevin®) to be an effective, safe control for burrowing shrimp (Final Environmental Impact Statement - FEIS, WDF 1985, and Supplemental Environmental Impact Statement - SEIS, WDF 1992.) Its use and effects have received continuing review and monitoring over the past 25 years, culminating in the preparation of the WDF's FEIS and SEIS. Concurrently, growers are in the process of developing an integrated pest management program for control of the burrowing shrimp. Control involves spraying carbaryl on beds during a single summer extreme low tide series. The application is strictly regulated and limited to 600 acres in Willapa Bay and 200 acres in Grays Harbor annually. Growers consolidate areas to be treated into large contiguous blocks of land, minimizing recolonization from adjacent infected beds. This method of control is only available to Washington growers. The marine application of carbaryl is not legal in Oregon or California. In these states, due to lack of effective controls, the burrowing shrimp have taken thousands of acres out of oyster production.

Burrowing shrimp control is a complex and controversial issue. While the shrimp population increase is generally acknowledged by all, the magnitude has not been documented. Various theories have been offered for the phenomenon, including fewer low salinity events in the estuaries because of climate changes and/or damming of the Columbia River. The shrimp are not tolerant of low salinities. Another possible factor is that the populations of salmon, which prey on larval stages of burrowing shrimp, and of sturgeon, which are uniquely suited to prey on adult burrowing shrimp, are severely depressed. Regardless of the cause, the burrowing shrimp have effectively turned thousands of acres of diverse, productive oyster and eelgrass habitat into desolate, largely monospecifc mudflats. The areas treated with carbaryl stabilize are seeded with oysters, and often are recolonized by eelgrass within the following year, providing a diverse habitat for a variety of invertebrates (Dumbauld and Wyllie Echeverria in press). Carbaryl treatments are generally required every 5 to 6 years to keep the ground in production.

Rototilling, mowing and harrowing. Some growers rototil beds prior to re-seeding to control oyster drills and SAV. Some growers also "mow" eelgrass growing on bottom culture oyster beds by towing "V" bars behind workboats. Controlling SAV prior to planting and during maturation improves growth and meat yield. Eelgrass and other SAV reduce water circulation and consequently the amount of food available to the oysters.

Seeding. Seeding involves substrate modification. "Mother" shells, with young oysters attached, are spread on the bottom.

Harrowing. In areas where oysters are prone to sinking or burying, growers tow spring harrows across beds to pull the oysters back to the surface.

Mechanical drag and hydraulic dredge harvesting of oysters. Oysters up and down the West Coast have been harvested for years with traditional drag dredges and to a lesser extent with (Hanks) hydraulic harvesters. Both of these methods disturb the substrate and eelgrass (or SAV) when present.

Clean-up dredging, rototilling, harrowing, and controlling SAV all have implications for juvenile salmon and their prey species depending on the time of year the activity is performed.

Substrate modification for clam culture. A number of Manila clam farmers in Puget Sound, and increasingly in Willapa Bay, add gravel to muddy substrate to improve clam productivity. Thom et al. (1992) determined there was an increase of certain salmonid prey species associated with the gravel substrate. However WDFW biologists are concerned the graveling activity can be disruptive to juvenile salmon during their out-migration between mid March and mid June. State biologists fear the graveling activity, which occurs at high tide, could scare juvenile salmon to deeper water where they are more susceptible to predation, or that important salmonid copepod prey species would be buried. Growers currently curtail graveling during the salmonid out-migration period.

The Sustainable Fisheries Act

The Sustainable Fisheries Act, which was passed by Congress in 1996 and amended the Magnusen-Stevens Fisheries Conservation and Management Act, requires the regional fishery management councils to identify essential fish habitat (EFH) for the species they manage in the exclusive economic zone (EEZ), typically 3 to 200 miles offshore. The councils were further directed by the Act to develop conservation and enhancement recommendations to protect EFH. The Pacific Fishery Management Council has developed EFH documents for salmonids, as well as for pelagic and demersal fish. The original draft of the salmonid EFH document was very controversial because of its misrepresentation of aquaculture impacts on salmon EFH, and proposed conservation and enhancement measures that would have impacted shellfish culture practices severely. Subsequent drafts have been modified to reflect potential impacts more accurately.

The shellfish industry's concern with both the ESA and the Sustainable Fisheries Act is that they trigger a federal consultation with the NOAA National Marine Fisheries Service (NMFS) if the activity requires a federal permit or if the company receives any federal funding. Historically, the majority of shellfish farming activities in the Pacific Northwest have fallen under a programmatic U.S. Army Corp of Engineers (USCOE) Nationwide Permit 4. Because it is a federal permit, the consultation process is triggered under both Acts. Growers now find themselves burdened by a whole new layer of regulatory bureaucracy by having to negotiate with NMFS on farming practices.

U.S. Army Corp Of Engineers Nationwide Permit 4 (NWP 4)

Recent changes in the NWP 4 permit are impacting shellfish culture operations in Oregon and California. Prior to 1991, the USCOE showed little interest in regulating shellfish culture activities: the NWP 4 permit covered oyster and clam digging. In 1991, new language was added to the NWP 4 that specified that oyster seeding was covered by the permit, provided it did not occur

in wetlands or vegetated shallows. In 1996, additional language was added clarifying that oyster seeding was covered by the permit so long as it did not occur in wetlands, in sites that support SAV, or in sites that have historically supported SAV even though it may not be present in that given year. Also, covered oyster trays and clam racks were singled out as no longer being covered by the NWP 4.

As a consequence of these NWP 4 changes, a number of shellfish culture operations will be required to get individual USCOE Section 10/404 permits. In Humbolt Bay, California, a large shellfish operator has been negotiating for an individual USCOE permit for 2 years, with the result that substantial changes to their historic farming practices are being required. Similarly, the Oregon Department of Agriculture is no longer allowing oyster leases in areas with SAV.

RESPONDING TO THE CHALLENGE

"Walking the Talk"

Recognizing the challenge posed by regulatory and environmental pressures, the PCSGA Board of Trustees is challenging growers to "walk the talk." The shellfish industry has long been recognized for its water quality advocacy efforts. Growers are regularly on the front line demanding clean water to grow their shellfish. If growers are going to demand that of everyone else, they should be willing to set a good example. Not only do growers need to be aware of their own potential impacts to water quality, they also need to understand where their farming operations are having negative environmental impacts and, where practical, be willing to mitigate those impacts. Conversely, where culture activities are providing a beneficial effect on the ecosystem, those activities should be recognized and promoted.

Get Involved

In addition to having the Executive Director dedicated to the issues, growers are encouraged to get involved locally in shoreline and growth management planning and also with watershed committees, Soil Conservation districts, and local environmental organizations having similar goals. Growers are also encouraged to be active in their communities. They are encouraged to sponsor sports teams, donate product to local fundraising events, adopt sections of highway or beaches for litter patrols, sponsor portable toilets in areas with high recreational use and the potential to impact growing waters, give farm tours, and give guest lectures at the local high schools and community colleges.

Promotion and Education

Besides these community activities, the industry participates in a variety of promotional and educational events over the course of the year. PCSGA sets up a raw bar in the Capitol Rotunda in Olympia for an afternoon during the legislative session to thank legislators and lobbyists for their continued support of water quality initiatives. Growers served free oysters to passers by in a courtyard adjacent to the Brooklyn Café and Oyster Bar in downtown Seattle on Oyster Appreciation Day. Anthony's Restaurants' Oyster Olympics and Elliot's Oyster House's Oyster New Years are two large annual promotions that raise money for and the public's awareness of clean water issues. Oysterfest, an annual festival in Shelton, Washington, draws over 20,000 people over 2 days and provides education on the benefits of shellfish and the need for clean water. The festival includes the West Coast shucking championship competition. The winner is sent to Maryland to compete in the national championship.

Environmental Management System (EMS)

With increasing regulatory scrutiny bearing down on the industry, the PCSGA recognized it would need a tool to encourage growers to "walk the talk." Patterned on the International Organization of Standards ISO 14,000 program, the PCSGA is developing an Environmental Management System. An Environmental Management System includes an Environmental Policy and an Environmental Code of Practice (ECOP) to implement the Environmental Policy. The ECOP equates to Best Management Practices (BMPs).

PCSGA growers first explored the concept of BMPs in the spring of 1997 with grant money from People for Puget Sound and the help of three students from the University of Washington's Environmental Management Program. Working with several growers, the students drafted lifecycle BMPs for the shellfish industry. At the end of the student's 3-month effort they had developed a product the PCSGA Board adopted as a draft (Ahlers et al. 1997). The Board committed to refining the BMPs to a document the whole West Coast industry could adopt and implement. An important goal of the University of Washington's Environmental Management Program, which encouraged industry support for the process, was to make companies more profitable, while at the same time, more environmentally responsible.

Since the completion of the draft by the students, PCSGA has been educating member growers about the importance of the ECOP. PCSGA has been coordinating with the British Columbia Shellfish Growers who are also interested in developing an ECOP. Paul Lupi, the executive director of the New Zealand Mussel Industry Council, was invited to speak at a Sea Grant workshop regarding their recently completed 3-year effort to develop the first shellfish Environmental Management System in the world.

The PCSGA Board recognizes that for growers to embrace and adopt an ECOP they needed to be involved in its development. The Pacific Shellfish Institute (PSI), the research arm of the PCSGA, has submitted proposals to the USDA Sustainable Agricultural Research Education and National Research Institute for funding to facilitate development of the ECOP along the West Coast. The goal is to include growers from all West Coast states, representing all cultured shellfish species and all culture techniques, in a bottom-up, multistakeholder process. Growers have approached NMFS and the Sea Grant Marine Advisory Program seeking their support in the effort. The Sea Grant Marine Advisory programs in Washington, Oregon, California, and Alaska have indicated an interest in assisting with facilitation of the process. A proposal has also been submitted to the PEW Charitable Trust Foundation for funding to support implementation of the ECOP.

Regulatory Stability

Natural resource industries lack regulatory stability in today's environmental climate. The rules governing business operations change frequently. This is true whether one is growing animals, row crops, trees, or shellfish. One of the few tools available for private landowners to achieve regulatory stability is the habitat conservation plan (HCP) process under section 10 of the Endangered Species Act. Growers have been discussing with NMFS the possibility of developing the ECOP into an umbrella HCP. This

could serve as a template for growers. Individual growers could prepare customized farm plans using the umbrella HCP as a template, and receive a "certificate of inclusion" under the umbrella plan. HCPs are generally 50-year contracts with the NMFS. The landowner agrees to land management that has protections for threatened or endangered species that often go beyond what current regulation might require. In return, the landowner gets an incidental "take" permit and regulatory certainty. The incidental "take" permit allows the owner to accidentally kill or harm the threatened or endangered species as long as they are operating within the provisions of their agreed contract. Without an incidental take permit, individuals can be subject to fines and/or imprisonment. This topic is of keen interest to Puget Sound shellfish farmers because, as noted earlier, the Puget Sound Chinook salmon were listed in March 1999 and the out-migrating juveniles utilize grower's beds in the transition from fresh to salt water.

Identifying Research Needs

An important aspect of developing an effective ECOP for shellfish cultivation is identifying the negative impacts in order to mitigate them effectively. Some impacts are understood, but many are not. The Pacific Shellfish Institute is conducting a literature review of existing research to identify needs for future research.

Goals 2010

At the PCSGA annual meeting in October 1998, a process was begun to set goals in eight research categories for the year 2010. With the goals identified, growers were asked to identify the research priorities and legislative initiatives necessary to achieve those goals. Shellfish ecology is one of the categories, with identified research priorities that attempt to fill gaps in existing knowledge and with the goal of minimizing negative impacts and enhancing positive impacts. The 2010 Goals, Research and Initiative Priorities, are available on the PCSGA website at www.pcsga.org. PSI is using the document to prioritize its research efforts as well as circulating it to the various research institutions, granting entities, and resource management agencies.

Industry, Scientist, Resource Manager Disconnect

As West Coast growers have become more proactive in the arena of shellfish ecology, it has become apparent that a segment of the research and resource management community is at odds with the industry. In the Chesapeake Bay on the East Coast of North America, it has been recognized that oyster reefs are an important part of the ecosystem, providing critical habitat and filtration to the estuary. Millions of dollars are now being spent in an attempt to rebuild oyster reefs in the Chesapeake Bay, not only to revive an important fishery but, more importantly, to restore the natural functions to the system.

On the West Coast, certain segments of the research and resource management communities have come to view eelgrass as the ultimate indicator of the health of an estuary. While it is a vital part of any coastal estuary, eelgrass is not the only indicator of a healthy ecosystem.

A consequence of the "eelgrass protectionist" philosophy on the West Coast has been a series of studies, frequently cited by resource managers, examining the effects of shellfish culture on eelgrass. When the studies conclude a negative impact of a culture activity on eelgrass, the resource managers have promoted regulations to prohibit the activity. The recent changes to the USCOE NWP 4 provide a good example. Unfortunately, the industry has been ineffective at getting the West Coast scientific community or resource managers to acknowledge the benefits of the shellfish to the ecosystem. In the East, hundreds of thousands of dollars of taxpayer's money tare being spent to restore oyster reefs; however, in the West, the regulatory screws continue to be tightened such that eventually farming shellfish will no longer be a viable economic enterprise.

Working with the Western Regional Aquaculture Center, housed at the University of Washington's School of Fisheries, the shellfish industry has recently secured funding for research that will investigate the oyster-eelgrass interaction to better understand the overall ecological role of the oysters in relation to the eelgrass. The Pacific Shellfish Institute is pursuing funding for similar studies related to suspended shellfish (oyster and mussel) culture.

The Ecological Benefits of Cultured Shellfish

Bivalves are efficient filter feeders. Oysters and other suspension-feeding bivalves play an important role in estuarine ecosystems as biofilters, significantly enhancing water quality and clarity, which have been the subject of studies in Chesapeake Bay (Gottleib and Schweighofer 1996). In fact, now that the oysters are virtually gone from the nutrient-rich Chesapeake ecosystem, algal blooms go largely unconsumed. Light cannot penetrate the algal blooms, and eelgrass and other submerged aquatic vegetation (SAV) are declining, as are the fish species dependent on the SAV for habitat and refuge.

Also, regarding the bivalve's filtering capacity, a recent (October 1997) report by the Environmental Defense Fund, "Murky Waters: Environmental Effects of Aquaculture in the United States" (Goldburg & Triplett 1997), noted that mollusk farming "actually reduces nutrient pollution. Mollusk farmers do not feed [their stock]. Clams, oysters, mussels and scallops are filter feeders that consume phytoplankton already in the water column. Mollusk culture actually reduces the nutrients in marine systems, because 35–40% of the total organic matter ingested by the mollusk is used for growth and permanently removed by harvest of the mollusk."

Oysters have been shown to have positive impacts on specific components of epibenthic communities and share many of the same attributes as eelgrass beds. Like other three-dimensional biological structures (including eelgrass beds and rubble reefs), oyster shells modify tidal flow and sedimentary processes and serve as important nursery and refuge habitats for juvenile fishes, shrimps, crabs, and other invertebrates (Ambrose and Anderson 1990; Doty et al. 1990; Breitburg 1991; Dumbauld et al. 1993; Williams 1994; Eggleston and Armstrong 1995; Simenstad and Fresh 1995). Interestingly, in Grays Harbor, Washington, the USCOE uses oyster shell to mitigate impacts to crab habitat from their dredging activities.

Doty et al. (1990) compared the abundance of intertidal juvenile crab in oyster, eelgrass, and open habitats and found that the oyster cover contained 4 to 6 times the crab abundance found in eelgrass. Basically, the open areas contained no juvenile crabs, and the eelgrass did not begin to play a protective role until mid June when it grew out. There is another important distinction between oysters and eelgrass relative to habitat: the oysters are there year-round. While eelgrass grows year-round, in the winter the blades are shorter, narrower, and fewer per shoot (Phillips 1984). Furthermore, many shellfish growers note that winter storms frequently eliminate almost all eelgrass in intertidal beds.

Graveling of tidelands and the broadcast of oyster shell are historic practices for enhancing shellfish production. Enhancement of secondary productivity, with increased standing stocks of epibenthic prey resources for juvenile salmon, as a result of intertidal graveling has been documented (Thom et al. 1992). The presence of oysters and gravel appear to have little, if any, adverse impact on species diversity and overall density compared to unaltered habitats, although shifts in species abundance and dominance may occur as a result of physical and biological modifications (Simenstad et al. 1991; Thompson 1995). Some of these shifts in species dominance may actually benefit outmigrating juvenile salmon. For example, Simenstad et al. (1991) found that densities of the harpacticoid copepod *Tisbe* spp., an important prey item for some juvenile salmonids (e.g., chum salmon, *Oncorhynchus keta*), were enhanced in areas of oyster culture and shell compared to bare mudflat. Brooks (1995) found that *Corophium acherusicum*, another critical prey resource for fish, was enhanced in actively cultured oyster beds, and data from Armstrong et al. (1992) indicate greater densities of gammarid amphipods and small tellinid clams as prey for both salmonids and 0+ Dungeness crabs (*Cancer magister*).

CONCLUSION

West Coast shellfish culture practices have recently come under greater environmental and public scrutiny as natural resource agencies and government officials direct more attention toward protecting estuarine ecosystems for their biological productivity, complex habitats, and diverse assemblages of aquatic species. The Pacific Coast Shellfish Growers Association is challenging shellfish growers to respond to the pressure by proactive involvement in their communities, through local planning, education, promotion, and research. As important, the PCSGA is working with growers and other stakeholders in the estuaries to develop an Environmental Management System for the shellfish industry. The EMS will establish an Environmental Policy and an Environmental Code of Practice for shellfish farmers up and down the West Coast. The industry is prioritizing research to better understand potential impacts as they develop an effective code of practice. Establishing shellfish growers as responsible users of the water surface, water column, and tidelands will be crucial to securing the future of the industry in the new millennium.

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A RESOURCE-BASED METHODOLOGY TO ASSESS DOCK AND PIER IMPACTS ON PLEASANT BAY, MASSACHUSETTS

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ABSTRACT The preparation of a four-town resource management plan for Pleasant Bay, Cape Cod, Massachusetts, required a comprehensive assessment of the present number of private piers on the bay, the probability of numerous additional piers in the future, and the potential impacts from piers on the resources of the bay. The planning group developed a methodology to account for various components relative to piers and their use. The study area was segmented into 26 geographic subsections. Each subsection was evaluated for nine factors representing biological, physical, and human use characteristics critical to the impacts of docks and piers. The nine factors were semi-enclosed or open water bodies, water depth, shellfish habitat, eelgrass, fringe salt marsh, density of existing structures, moorings, and navigational channels, and recreational activity. After assessment of the areas, each of the nine factors was assigned a value of 0, 0.5, or 1, where 0 indicated the least significance and 1 represented the greatest significance. Results were tabulated and mapped according to resource sensitivity. The results indicated that a significant portion of the bay's more secluded shoreline is extremely resource sensitive. The environmental impacts from construction and use of docks and piers in these areas pose a direct threat to the extensive and fragile resources, and these areas have been deemed inappropriate for new docks and piers. Less sensitive areas may be more appropriate for construction of new docks and piers based on baywide criteria to be formulated once the plan is implemented.

KEY WORDS: Docks, piers, resource assessment, boating impacts, management

INTRODUCTION

To a shorefront property owner, one of the primary reasons for paying higher taxes for waterfront property is the immediate access to the water. A dock in front of a house represents easy access to the water, status, a higher property value for re-sale, and a property right. The structure is generally located in public waters in Massachusetts if it extends below the high water mark. To a resource manager, a dock represents a potential conflict with resource protection, actual negative effects on the marine environment, and private intrusion in public waters that is not a private property right. These conflicting representations often result in frustration to both the homeowner and manager because there is generally no cohesive policy toward the siting of new docks. While the structures and their use are cause for concern, the cumulative impacts have not been researched adequately. As a result, in developing the Pleasant Bay Resource Management Plan (known as the Bay Plan), we have taken a conservative approach toward the siting of new docks based on our own observations as well as information provided by other researchers.

Although generally thought of as benign structures in the intertidal and subtidal zones, docks and piers (hereinafter referred to as docks) have been shown to be potentially problematic. Concern centers on the effects of docks in shallow embayments and includes vegetation loss from shading, shellfish habitat loss, impacts to eelgrass, chemical leachates from treated wood, construction impacts, fragmentation of beach habitats, sediment resuspension from boat propellers, boat paints, chemicals used in marine sanitation devices, and petrochemicals.

Arguments used by homeowners or their agents to gain a permit for a new dock suggest that docks can have a beneficial environmental effect. Carriker (1961) stated that a piling could create a microhabitat, changing the overall circulation patterns such that a small gyre is created, allowing for better settlement of shellfish larvae. How multiple docks in an area affect currents is not well understood. Untreated pilings are known to attract settlement of certain marine organisms. Another argument we have heard is that a dock is better than a boat on a mooring because less total habitat may be affected. A boat at a dock is at a fixed location, while on a mooring the mooring chain may scour the bottom as the boat swings (Walker et al. 1989, Short et al. 1993). Also, a dock crossing over a marsh is better than a path through a marsh because there is less total impact on the marsh vegetation.

Negative effects of the structures can include physical displacement of habitat from pilings, pipes, or other upright structural members. While not usually perceived as a great threat, the cumulative effect of the number of pilings per dock multiplied by the number of docks can be substantial. Docks can shade submerged aquatic vegetation (SAVs) (Wetzel and Penhale 1983, Short et al. 1993; Burdick and Short 1995). Seasonal docks that are removed

yearly can cause dead zones of anoxic sediment in some areas that is up to twice the diameter of the pilings (Macfarlane personal observations). However, permanent docks that remain in the water during the winter can be lifted by ice, requiring reinstallation of the pilings and reducing the amount of accessible shellfish habitat for harvest. Use of pressure-treated wood can be toxic to marine organisms (Weis et al. 1991; Weis and Weis, 1992a,b, Weis et al. 1993). Lastly, there is an aesthetics issue regarding the value of natural shorelines versus those having manmade structures.

Operation of the boats tied to docks can cause problems (Crawford et al. 1994). Prop dredging can mechanically remove habitat while also causing resuspension of sediment. Although turbidity is a natural phenomenon in wind-driven resuspension episodes, turbidity caused by boats has not been well studied and is poorly understood (Yousef 1974; Yousef et al. 1980, Hilton and Phillips 1982). The effect of petrochemical spills and chronic addition of petrochemicals to the water from boats operating at a fixed location is also poorly understood as is potential damage from boat paints, especially formulations of bottom paint.

CURRENT PERMITTING SYSTEM

Under the current permitting system in Massachusetts, a property owner desiring a new dock must apply to three agencies for three separate permits. First, the homeowner needs an Order of Conditions from the local Conservation Commission, the Selectmen-appointed body of volunteers responsible for administering the state Wetlands Protection Act (MGLC.131) and local wetlands bylaw (if applicable). Second, a Chapter 91 (Waterways) license from the Massachusetts Department of Environmental Protection Waterways Program must be obtained. (In Massachusetts, the public has additional rights of fishing, fowling, and navigation in the intertidal zone. These latter rights are part of the public trust doctrine and were established in the Massachusetts Colonial Ordinance of 1641–1647.) Third, a permit must be obtained from the U.S. Army Corps of Engineers in accordance with Section 404 of the Clean Water Act (Federal Water Pollution Control Act of 1948).

In accordance with Massachusetts' amended waterways regulations (Chapter 91), no new dock could be permitted in any area designated by the State as an Area of Critical Environmental Concern (ACEC) until and unless the town completed a state-approved resource management plan. Pleasant Bay was designated as an ACEC in 1987. As a result, there has been a moratorium on the issuance of new dock permits since 1991. At the time the moratorium went into effect there were 165 docks in the Pleasant Bay estuary, the majority of which were located in the more protected areas of the bay.

The performance standard in the existing regulations for allowing docks in an ACEC is "no adverse effect." Prior to the moratorium, property owners applying for dock permits would institute mitigating measures to counteract the potential problems outlined above. Shading effects can be overcome by raising the structure to sufficient height to allow for sunlight to reach the grass, adding "holes" (through greater plank spacing) in the walkway for sunlight penetration, or using plastic or metal grates. Permanent structures can be maintained by use of bubbler systems during the winter months of ice. Shellfish can be seeded around the dock. Pressure-treated wood can be replaced by plastic "wood," cedar, locust, or other nonleaching materials. To many conservation commissions, these measures, presented as mitigation by homeowner representatives (engineers, consultants, and lawyers), were enough to maintain the standard of no adverse impact. In addition, once one person received a permit, it was difficult to identify an adjacent property as too fragile to permit a dock and the neighbor generally received a permit as well. Moreover, each application was judged on its own merits and although cumulative effects were an interest protected by the state Wetlands Protection Act, these cumulative effects were difficult to prove in potential court appeals.

New Jersey approached the problem of docks proliferation through development of a generic Environmental Impact Statement (Crawford et al. 1998). Because EIS provisions are lacking in Massachusetts, the Pleasant Bay Technical Advisory Committee (TAC) developed a methodology to look at the proliferation of private docks in a different manner. The Bay Plan was based not on individual lot-by-lot decisions or a generic basis, but rather by a method for estimating cumulative impacts and determining where docks could be built that would not have an adverse impact on the system as a whole.

STUDY AREA

Pleasant Bay is located at the outer part of the elbow of Cape Cod within the towns of Chatham, Harwich, Brewster, and Orleans (Fig.1). Designated as an ACEC in 1987, over 9000 acres (3645 ha) are within the boundary. Chatham Harbor is not within the boundary of the ACEC, but it is a major part of the estuary since the inlet for the bay is in the harbor. The primary reason for the harbor's exclusion from the ACEC designation was that Chatham is home to the region's offshore fishing fleet and, in order to maintain that industry, dredging is sometimes required, an activity prohibited by the designation. However, the harbor was included for the purposes of developing the management plan. Brewster has a mere 40 ft (12 m) of shoreline, but it contains the largest portion of the bay's watershed.

The bay is a shallow embayment, where over 50% is less than 2 m deep at mean low water (MLW). It has a migrating barrier beach on its eastern flank that protects the bay from the Atlantic Ocean. The shallow portions of the Little Bay have healthy eelgrass (*Zostera marina* (Linnaeus)) meadows and sometimes produce bay scallops (*Argopecten irradians irradians* Lamarck). The habitat can support clams (*Mya arenaria* Linnaeus) and quahogs (*Mercenaria mercenaria* Linnaeus 1758). Mussels (*Mytilus edulis* Linnaeus) are frequently found near Chatham Harbor. Oysters (*Crassostrea virginica* Gmelin) have not been a native species for at least the last 50 years, although they have been found sporadically as a direct result of a privately leased oyster area in the bay. The bay is ringed with fringe salt marsh, primarily salt marsh cordgrass (*Spartina alterniflora* (Loisel)). There are numerous pockets of larger salt marshes indenting the shoreline. Coastal banks are the dominant land interface feature.

PLEASANT BAY RESOURCE MANAGEMENT PLAN

Although the state imposed a moratorium on the construction of any new docks in Pleasant Bay, the towns were slow to get together to develop a joint plan. Several attempts were made and finally, in 1995, the four towns entered into an intermunicipal Memorandum of Agreement to develop a joint resource management plan to protect the extensive resources of Pleasant Bay. The resulting plan was approved in Harwich in May 1998 and by

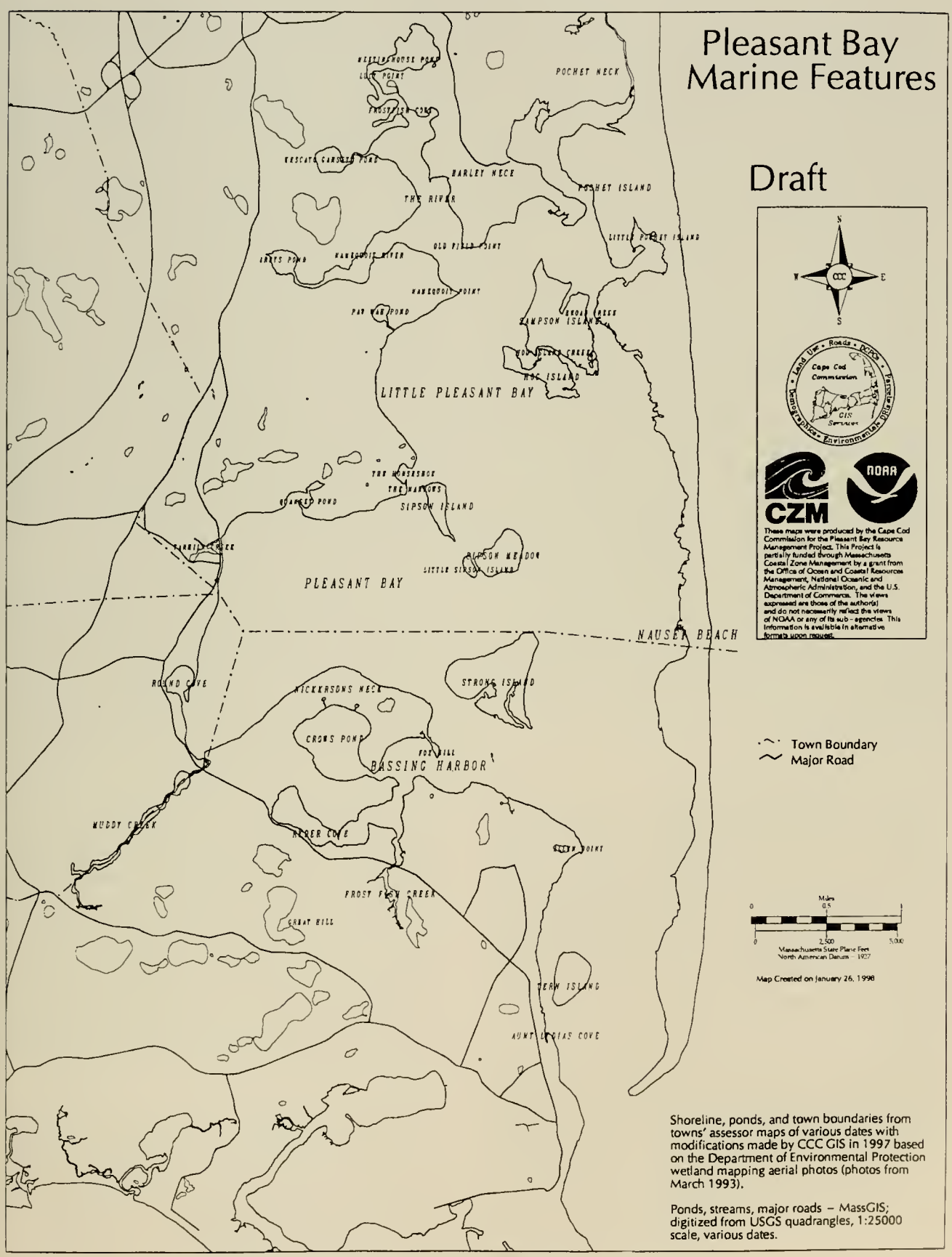

Figure 1. Map of the Pleasant Bay Resource Management Plan study area.

Orleans and Chatham in November 1998. It was rejected by Brewster in May 1998. However, even with Brewster's action, the plan can still be implemented since many of the recommendations centered on use of the water and the directly adjacent waterfront.

One of the primary reasons for the towns to get together was the dock issue. Each town had received numerous requests for docks since the moratorium had been put into effect. Orleans alone received at least 20 requests per year (Macfarlane personal observation), most of which emanated from new property owners. As the economy improved during the 1990s, it became widespread knowledge from realtors that a dock added anywhere from $50,000 to $100,000 to the value of a home on Pleasant Bay.

From 1995 to 1998, the towns worked together to develop the plan. This was the first time such a large cooperative effort was achieved in the history of the towns. The Selectmen in each town appointed a steering committee representative. A technical advisory committee was established, comprising shellfish constables, conservation administrators, harbormasters, water quality specialists, and town planners from each town. A consultant was hired as a coordinator. State and county governments and National Park Service (from the Cape Cod National Seashore) personnel served on the Technical Advisory Committee (TAC) as well.

The planning process revolved around five issues: structures (docks and coastal engineered erosion control structures), shellfish and aquaculture, boating, biodiversity, and public access. During the winter of 1997, the Steering Committee sponsored workshops on each issue (several hundred people participated) to define problems and make recommendations for the plan.

The Structures Workgroup reviewed the current statutes, bylaws, and regulations of dock applications and permitting. Given the history of permits approved prior to the moratorium, this group of the TAC was frustrated by the lot-by-lot approach and the lack of a generic EIS and agreed that there had to be another way to approach the subject.

THE METHODOLOGY

The TAC compiled data to create maps on a GIS system with the assistance of the Cape Cod Commission, a county planning and regulatory agency. Base maps were created for land use, location of shellfish resources by species, marshes and eelgrass, existing docks and mooring fields, town landings, navigational channels, scenic view points, access points, and all other resources. From the base maps, overlays were made to determine conflicts. In Pleasant Bay, shellfish are often located in a thin ribbon of land between the edge of the fringe marsh and about 250 ft (76 m) offshore, in both intertidal and subtidal lands. It is also the location of most of the docks.

When the map of the docks was overlaid onto the map of shellfish, the upper part of the River Complex in Orleans (Meetinghouse Pond to Namequoit Point) and the protected areas of Crow's Pond and Ryder's Cove in Chatham exhibited the most overlap. When moorings were added, it was obvious that boating activity heavily impacted these upper areas. The question posed was, Would these areas remain productive for shellfish if there was a dock every 150 ft (45 m), the average lot frontage?

The approach of the TAC was to look at the bay as a system. Constructing a dock in a salt pond at the far end of the estuary was far different in terms of impact than constructing a dock on the open shoreline along either Little Bay or Big Bay. A method was needed that could distinguish the differences between these two areas and so a sensitivity index was developed. The process was divided into ten steps, discussed below.

Step 1: The bay was divided into 26 segments or subsections that were different from one another in one or more ways. These subsections included pond, river, and open bay shorelines (Fig. 2).

Step 2: The attributes of each subsection were described with respect to biological, physical, and human use factors (Fig. 3). Did the area support shellfish? Did the area have eelgrass and/or fringe marsh? What was the depth of water 200 ft (60 m) from the edge of the marsh or MLW, whichever applied? Was the area open water, a river, or semi-enclosed? What was the ratio of docks to parcels without docks? Was there a navigational channel within 500 ft (150 m) of shore?

We chose these questions because in total they would give us a sense of the area and what the potential impacts of a dock would probably be. Because aesthetics is a difficult and subjective concept to quantify, we asked ourselves what was it about a dock that produces a reaction when looking at them? The answer to us was that docks make a natural shoreline look manmade. The greater the number of docks within a visual path and the higher the docks were, the less aesthetically pleasing the scene was. We judged natural shorelines to be highly aesthetic and manmade structures to be less aesthetic. Therefore, we were able to address the aesthetics by using the ratio of parcels with docks to those parcels without them.

Step 3: Nine criteria for the sensitivity index were established and each criteria was evaluated according to one of the following: high, medium, low; shallow, medium, deep; lots, some, none; yes, some, no. A matrix was developed using these criteria for each of the 26 areas (Table 1). The nine criteria were open or semi-enclosed water body; ratio of parcels with docks to those without; shellfish habitat; fringe marsh; eelgrass; water depth within 150 ft (45 m) of shore; moorings within 500 ft (150 m) of shore; navigational channel within 500 ft (150 m) of shore; recreational use.

Step 4: Terms were defined (Table 2).

1. Semi-enclosed or open water body: A semi-enclosed area, such as a salt pond at the head of the estuary, would have lower flushing capacity and would be the first area to show signs of problems resulting from nutrient loading, drainage, or toxic inputs. An open area would have greater exchange with the incoming oceanic water.
2. Dock ratio: Number of lots with docks and number without.
3. Shellfish habitat: The shoreline was evaluated with respect to the historical, present, and town propagation projects to determine an area's ability to support shellfish. It was also evaluated with respect to populations of clams, quahogs, and scallops; mussels and oysters were not present in the waters in the 200-ft (60 m)-wide shellfish area adjacent to the shore.
4. Fringe marsh: Present in many areas, fringe marsh varies considerably in width. It protects coastal banks from erosion and, while not as productive as extensive marshes, fringe marsh has the capacity to filter nutrient-loaded groundwater, contains invertebrates such as ribbed mussels (*Modiolus demisses*) that can aid in water clarity, and provides habitat for fish and invertebrates. It has widths varying from minimal to about 20 ft (6 m), with 10 to 20 ft (3–6 m) being average. A

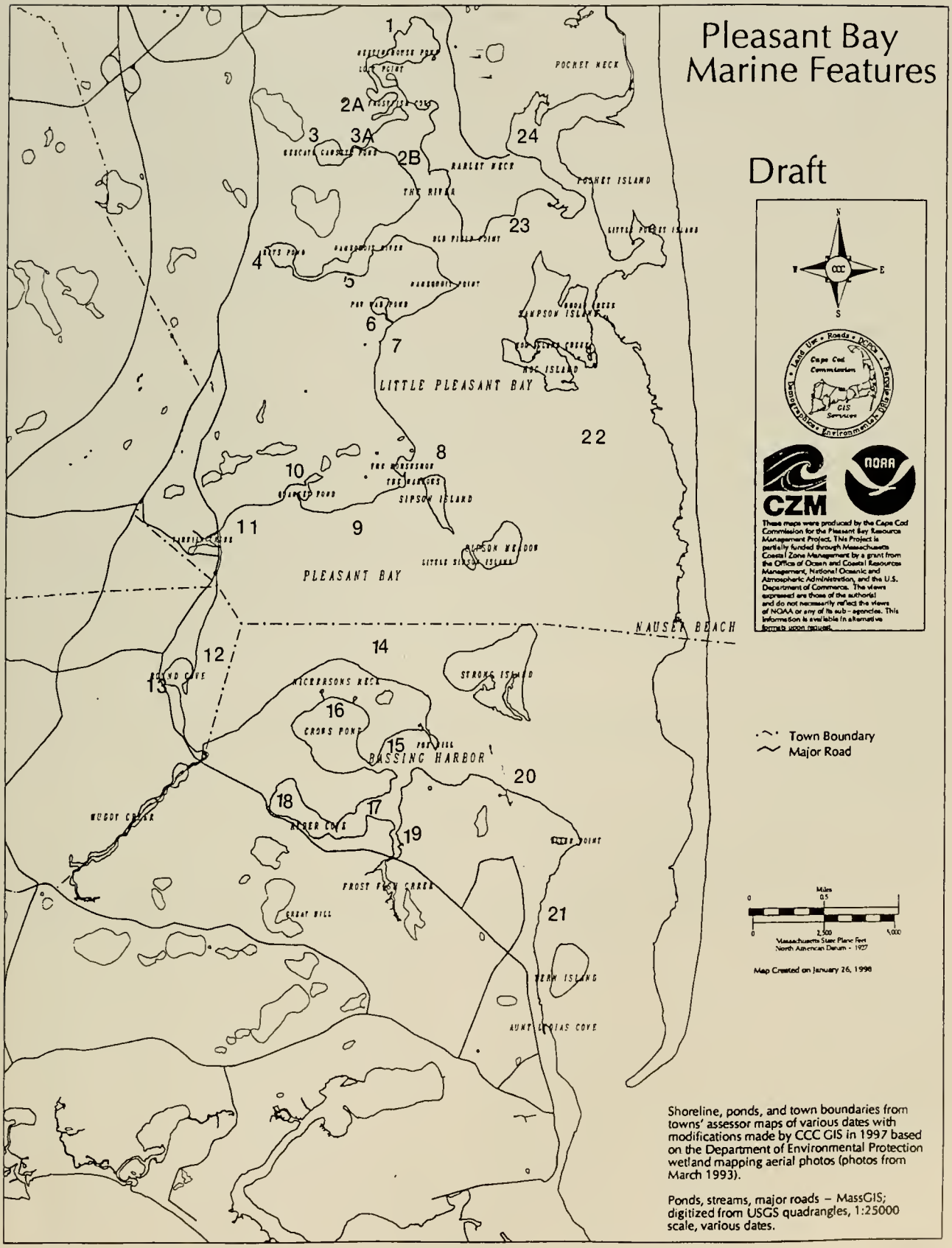

Figure 2. Map of the Pleasant Bay Resource Management Plan subsections.

PLEASANT BAY: SUBSECTION AREA DESIGNATIONS AND DESCRIPTIONS

1. *Meetinghouse Pond* is an enclosed pond on the upper end of an estuary. If there is a problem with flushing in the bay, it would be most noticeable here. There is a low dock to parcel ratio currently, with less than 25% of the parcels with docks, which means approximately 75% of the parcels could potentially each request a dock. It is a known shellfishing area close to shore (approximately 200 feet from fringe marsh) and beyond, which would coincide with the locations of potential dock placements. There is some deep water shellfishing here, but not in the fringe area. Fringe marsh may be found along the entire shoreline, while eelgrass is spotty, and the water depth is shallow (shown in bathymetry results). The pond is heavily used for moorings, it has no navigational channel, but is highly used by people coming and going for recreation. It is a busy place possibly due it's having both filling and pump-out stations.

2. *Kent's Point—Upper River*, which as a river makes the designation of enclosed versus open a difficult one. It is a narrow body of water, where over 50% of the parcels have docks. This is a known shellfish area with a productive fringe marsh. There is spotty eelgrass, the area is shallow, moorings are medium density, there is definitely a navigational channel and is a busy area for its size.

3. *Kent's Point—Namequoit* is less narrow than the Upper River although it is very narrow around Mayflower Point. This area has a medium density of docks to parcels, and is a known spot for shellfishing, although some parts are not highly productive. There is fringe marsh in the majority of this area, although spotty in some locations, and it has a medium density of moorings, definite navigational channel and heavy recreational use.

4. *Lonnie's Pond* is definitely enclosed with a narrow channel leading to it. (This is a potential site for eutrophication in the future.) There is a medium density of docks to parcels, known shellfish habitat in the fringe and deep waters along the fringe, spotty eelgrass, deep water close to shore, heavy use of moorings, no navigational channel and heavy navigational use.

5. *Lonnie's Channel* is narrow and shallow. There is one dock, some shellfishing in the river itself, fringe marsh throughout the channel, no eelgrass, and heavy recreational use from people coming and going.

6. *Arey's Pond* is similar to Lonnie's pond and very active.

7. *Namequoit River* may be described similarly to Lonnie's Channel except that the channel is wider which provides more space for moorings.

Figure 3. Descriptive attributes of each Bay Plan subsection.

TABLE 1.

Inventory of resource and use issues relevant to docks and piers.

Area	Number of Docks	Parcels w/ No Dock	Shellfish Habitat	Fringe Marsh	Eelgrass	Water Depth w/in 150'	Moorings w/in 150'	Navigational Channel w/in 150'	Recreational Use
(1)									
Meetinghouse Pond	11	32	Yes	Heavy	Light	Shallow	Heavy	No	Light
(2A)									
Kents Point-Upper River	18	10	Yes	Heavy	Light	Shallow	Light	Yes	Heavy
(2B)									
Kents Point-Namequoit	19	39	Yes	Heavy	Heavy	Shallow	Light	Yes	Heavy
(3)									
Lonnie's Pond	8	8	Yes	Heavy	Light	Deep	Heavy	No	Light
(3A)									
Lonnie's Channel	1	6	No	Heavy	None	Shallow	None	Yes	Heavy
(4)									
Arey's Pond	8	12	Yes	Heavy	Light	Shallow	Heavy	No	Light
(5)									
Namequoit Riv	16	32	Yes	Heavy	Light	Shallow	Heavy	Yes	Heavy
(6)									
Pah Wah Pond	6	7	Yes	Heavy	Light	Deep	Heavy	Yes	Light

TABLE 2.

Definition of terms.

Shellfish Habitat: All traditionally state-regulated species (soft shell clams, quahaugs, mussels, scallops and oysters) that have historically, currently or in the future could potentially support shellfish.
Shellfish: All species are of equal value.
A. Yes = evidence of being able to support shellfish
B. Slight = supports shellfish but not in abundance
C. No = no evidence of supporting or being able to support shellfish
Fringe Marsh: Bands of Spartina alterniflora with some S. patens in transition areas from the water to the upland.
A. Heavy = 10 feet or greater in width of marsh grass
B. Medium = 5–10 feet width
C. Light = <5 feet width
Eelgrass: Amount of eelgrass in a particular area
A. Heavy = sediment covered with eelgrass with few bare spots
B. Medium = eelgrass interspersed with bare sediment of equal proportions
C. Light = no eelgrass or a few sporadic individual plants
Ratio of docks: Number of lots with docks compared with total number of lots in a given area
Water Depth:
A. ≥4 feet at Mean Low Water (MLW) = low sediment disturbance
B. 3–4 feet = medium disturbance
C. <3 feet = high disturbance
Moorings: Public mooring area where density of moorings exceeds three moorings within 500 feet from shore.
A. Heavy = more than 3 moorings or mapped public mooring field
B. Light = 0–3 moorings per land parcel
C. None = no moorings in area
Navigational Channel: Structures placed less than 500 feet from channel would cause impediments to navigation.
Yes = channel within 500 feet; No = channel greater than 500 feet
Recreational Activity: General public use of the area
A. High = heavy use usually from boating activity
B. Medium = some boating or other water use
C. Light = very little boating or other water use

marsh 5–10 ft (1.5–3.0 m) wide was considered to provide some functions, but one that was less than 5 ft (1.5 m) wide probably provides only minimal positive effects.

5. Eelgrass: The presence or absence and relative abundance of eelgrass was assessed. The healthy meadows in the middle of Little Bay were considered as representative of heavy abundance, while a few sporadic plants were considered low abundance.
6. Depth of water: A depth of 4 ft (1.2 m) or more would have relatively little resuspension of particles from most outboard engines on most shallow-draft boats (Crawford et al. 1998). A boat put in gear and docking on the return trip can have an enormous impact and can even create a channel or hole known as "prop dredging." A depth of 3–4 ft (0.9–1.2 m) a boat would cause medium disturbance and at a depth of less than 3 ft (0.9 m) at MLW could cause resuspension.
7. Moorings: The harbormasters automatically give waterfront property owners up to three moorings in front of their property. A mooring field is a public mooring area where moorings exceeded that density within 500 ft (150 m) of the shore. Areas near town landings had such mooring fields while other areas generally did not.
8. Navigational channel within 500 ft (150 m): Many areas are highly used for both recreational and commercial activity. In accordance with state statute, a private structure should not be an impediment to navigation. Current channel locations were located and it was determined that structures less than 500 ft (150 m) from the channel would cause an impediment from wakes, and cross-channel operation.
9. Recreational activity: Although difficult to actually define, the area was examined with respect to the general public use of the area. A long, narrow river connecting to a pond with a lot of boats would have a lot of recreational activity near shore, as would most of the ponds because of the heavy number of moorings. The open water where docks would be located did not have much activity.

Step 5: We added a number to the descriptions as follows:
1. high, yes, lots, shallow = 1
2. medium, some, few = 0.5
3. low, no, deep = 0

Step 6: The words in our matrix were replaced with the numbers for each criterion at each location in a second matrix (Table. 3).

Step 7: The numbers were analyzed with respect to the total impact of docks and their use in the particular segments of the bay. Areas with the highest sensitivity were deemed to be inappropriate for new docks; areas with medium sensitivity may be able to support new docks with additional criteria; areas of low sensitivity could potentially be opened for new dock applications.

Step 8: The data were mapped and analyzed and the cut-off points determined (Fig. 4). Anything greater than 5.5 was deemed to be highly sensitive.

Step 9: Additional criteria were developed for the medium and low sensitivity areas for conservation commissions throughout the

TABLE 3.

An assessment of biological, physical, and human use impacts on the shoreline of Pleasant Bay (7/97)

#	Section	Area	Enclosed	Docks	Shellfish	Fringe	Eelgrass	Water Depth	Moorings	Navigat Ch	Recreat.	TOTAL	Ranking
1	9	Bay/North Shore	0	0	0.5	0	0.5	1	0	0	0.5	2.5	Less
2	7	Little Bay	0	0	0.5	0.5	0.5	1	0.5	0	0.5	3.5	Sensitive
3	23	Old Field Pt	0	0	0.5	1	0.5	1	0	0	0.5	3.5	
4	21	Chatham Harbor	0	0	0.5	0	0	1	0.5	1	1	4	
5	11	Bay/Northwest	0	0.5	0.5	0	0.5	1	1	0	1	4.5	
6	12	Bay/West	0	0.5	0.5	0.5	0	1	1	0	1	4.5	
7	22	North Beach	0	0	1	1	1	1	0	0	1	5	
8	3A	Lonnie's Channel	1	0	0.5	1	0	1	0	1	1	5.5	
9	14	Nickersons Neck	0	0	0.5	0.5	1	1	0.5	1	1	5.5	
10	19	Frost Fish Creek	1	0.5	1	1	0.5	1	0.5	0	0	5.5	
11	20	Ministers Pt	0	0	0.5	0.5	1	1	0.5	1	1	5.5	
12	1	Meetinghouse	1	0	1	1	0.5	1	0.5	0	1	6	
13	3	Lonnie's Pond	1	0.5	1	1	0.5	0	1	0	1	6	to
14	16	Crows Pond	1	0.5	1	0.5	1	0	1	0	1	6	
15	8	Narrows, Sipsons	0.5	0.5	1	0.5	0.5	1	0.5	1	1	6.5	
16	24	Pochet Inlet	1	0	1	1	0.5	1	0.5	1	0.5	6.5	
17	4	Arey's Pond	1	0.5	1	1	0.5	1	1	0	1	7	
18	6	Pah Wah Pond	1	0.5	1	1	0.5	0	1	1	1	7	
19	10	Quanset Pond	1	0.5	1	1	0.5	0	1	1	1	7	
20	13	Round Cove	1	0	1	1	0.5	0.5	1	1	1	7	
21	18	Ryders Cove	1	0	1	1	0.5	1	1	1	0.5	7	
22	15	Bassing Harbor	1	0	1	1	1	1	0.5	1	1	7.5	
23	2A	Kent's Pt-Upper	1	1	1	1	0.5	1	0.5	1	1	8	
24	2B	Kent's Pt-Nam	1	0.5	1	1	1	1	0.5	1	1	8	Most
25	5	Namequoit Riv	1	0.5	1	1	0.5	1	1	1	1	8	Sensitive
26	17	Upper Ryders	1	0.5	1	1	0.5	1	1	1	1	8	

Biol., Phys., Human Use Values = 0, .5, 1 TOTAL (Sum) = 0 to 9

region to adopt in their local regulations, which will be done through implementation.

Step 10: As the final step, the method was presented to the public.

CONCLUSIONS

The Bay Plan included the methodology (described in its appendix). The public was made aware that the moratorium will continue in the River Complex, Crow's Pond, Ryder's Cove, Quanset Pond, Round Cove, Pau Wah Pond, the Narrows, and Pochet. The plan was adopted in November 1998, and though many seasonal residents were not available to comment on the plan at that time, negative comments from them were minimal when they returned. Additional criteria were developed by the Technical Resources Committee (renamed after plan adoption) for areas where docks may be permitted in the future. The Orleans Conservation Commissions adopted the method and additional criteria in December 1999 by formalizing them in their regulations; Harwich and Chatham have not done so yet. The Bay Plan received a vote of approval from state agencies as written, including the dock and pier methodology.

As far as we have been able to ascertain, the methodology described herein is the first such attempt to address the environmental problems associated with private docks that was based on a baywide approach. As resource managers, we often have to make policy decisions based on incomplete information. We eagerly anticipate results from research that further amplifies our knowledge regarding the impacts of docks and their use on marine environments.

By eliminating the lot-by-lot procedures, we have also eliminated a more subjective approach to the permitting procedure. Using biological, physical, and human use parameters over wider areas has resulted in a management plan that will be more difficult to refute on appeal. However, we are willing to adjust the bottom-line recommendations should research prove that our designations have been overly conservative. Our charge in developing a resource management plan was to protect the bay. The following statement in the introduction to the plan sums up our approach:

> *An estuary left alone will nurture and care for itself with no help of human hands. It is only when human activities interfere with natural processes that the Bay responds by showing signs of stress, damage and disease. This stewardship plan for Pleasant Bay is based on the premise that human intervention in the natural processes of the Bay must be minimized. With this premise, the plan seeks to encourage a level of human use which does not upset the balance of the Bay's ecosystem, endanger the productivity of its wildlife, or invade the tranquility of those who seek its shores. Accomplishing this will require residents, visitors and commercial interests alike to place the long-term health of the Bay above individual interests. It will require change and sacrifice, and on-going commitment to preserving the health, beauty, and tranquility of Pleasant Bay for future generations.*

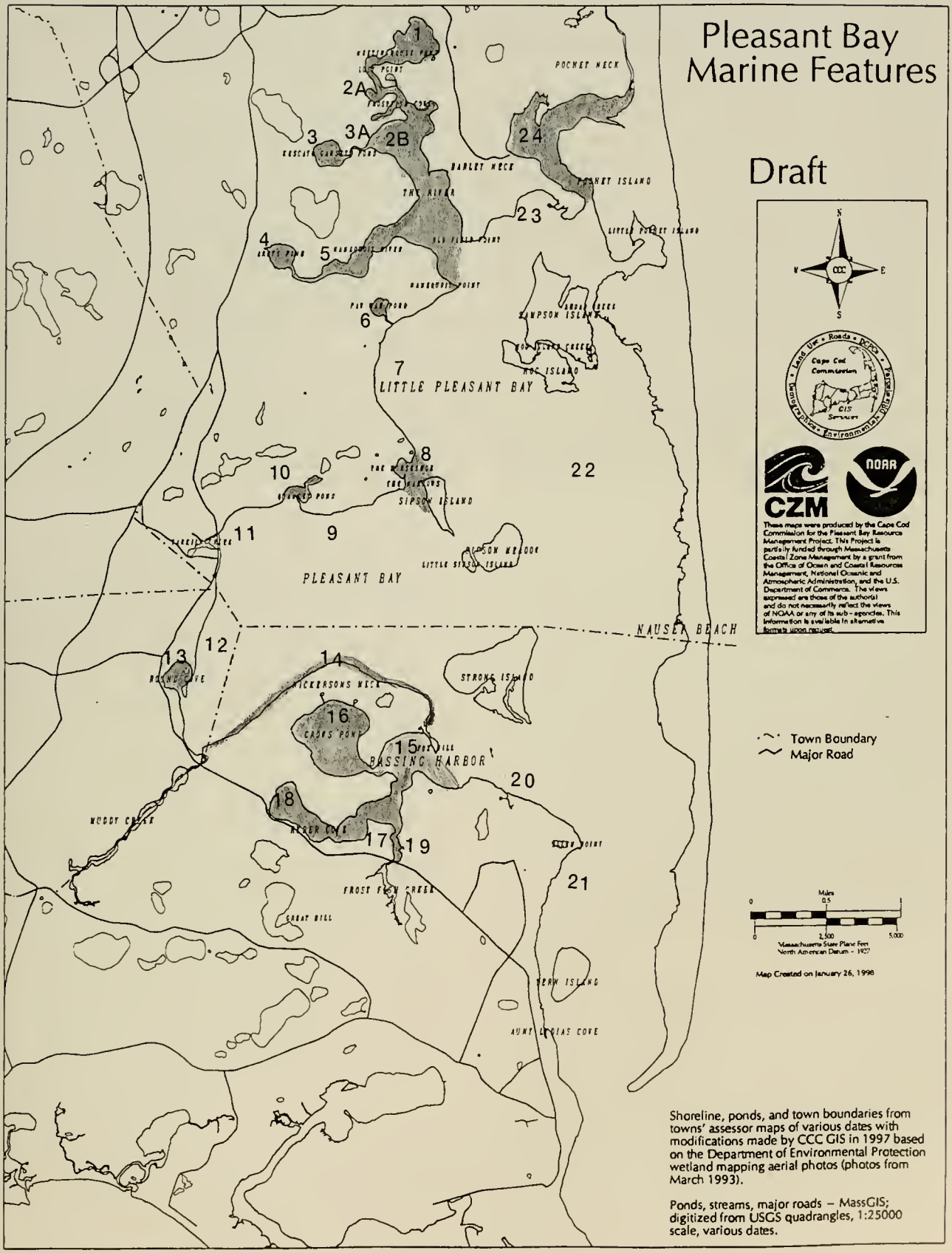

Figure 4. Map of the Pleasant Bay Resource Management Plan subsections based on the numerical sensitivity index.

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POOR WATER QUALITY? NOT IN MY BACKYARD! THE EFFECTIVENESS OF NEIGHBORHOOD POND ASSOCIATIONS IN THE PROTECTION AND IMPROVEMENT OF SHELLFISH GROWING WATERS ON MARTHA'S VINEYARD

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ABSTRACT Neighborhood pond associations have proven to be an especially effective force in the protection and enhancement of local coastal ecosystems. Environmental managers who are wise enough to forge partnerships with these local organizations will be rewarded with the energy, commitment, and passion reserved for issues that hit close to home. With the vigilance and dedication of a Neighborhood Crime Watch, local pond associations are the eyes and ears that sound the first alerts of environmental pollution. With the efficiency of local Minutemen, they rally to the cause as volunteer environmental advocates who offer financial commitment, a wealth of expertise, and effective political organization.

The environmental accomplishments of neighborhood groups on Martha's Vineyard are impressive. The Edgartown Harbor Association funded a water quality study resulting in the establishment of a free sewage pump-out facility for boaters. The Friends of Sengekontacket (Pond) provided the leadership to coordinate a local, state, and federal partnership to complete a major dredging project that restored filled shellfish habitat and nourished an eroding barrier beach. The Lagoon Pond Association funded a court battle to limit pier construction. The Tisbury Great Pond Think Tank and Tisbury Waterways, Inc. have addressed farm and roadway runoff with fencing, buffer strips, and innovative catch basins. All have conducted successful public education programs, water quality monitoring studies, and fundraising activities designed to protect shellfish habitats and water quality. Many have advanced protective zoning initiatives.

KEY WORDS: Martha's Vineyard, water quality, neighborhood pond associations

Located about 7 miles off of Cape Cod in southeast Massachusetts, the 100 mi^2 island of Martha's Vineyard has historically had an economy based on fishing and farming. Its rural seaside ambiance has made it a popular tourist destination; so much so, that now 95% of the island's economy is tied to tourism, seasonal residents, and vacation home development. In the past 20 years alone, the year-round and summer populations have doubled to 12,200 and 72,600, respectively. On a big holiday weekend, police estimate that the population can soar to nearly 150,000.

This recent dramatic increase in human population and activity threatens the shellfish resources. The increases in the number of houses, boats, piers, and paved roads have taxed the island's natural systems. Ground and surface waters are at increased risk from bacterial, chemical, and nutrient pollution, and the growth in boating and the associated construction of piers are destroying shellfish habitat. Preserving and enhancing shellfish resources in the wake of this explosive development is a monumental challenge.

The historical importance of the local shellfish industry has been a key factor in its preservation. On Martha's Vineyard, shellfishing is a cultural icon on par with the proverbial motherhood and apple pie. It should come as no surprise that when shellfish beds began to close due to high fecal coliform counts, the populace of Martha's Vineyard rallied. Neighborhood pond associations were formed and/or strengthened in response to developments that threatened the island's shellfish resources. Due in part to their vigilance, a remarkable 98.8% of the Island's 176,261 acres of shellfish waters are approved for harvest. Neighborhood pond associations are active participants in the protection and management of all the significant shellfish growing waters on the island. These associations have proven to be an especially effective force in the protection and enhancement of local coastal ecosystems.

Although some associations existed as social organizations previously, the majority of the organizations formed in the late 1980s and early 1990s in direct response to the environmental problems that followed the building boom in the mid 1980s. Water closures due to fecal coliform and proposed condominiums and subdivisions were the impetus for the formation of the groups. The Chilmark Ponds and Lagoon Pond associations are primarily composed of waterfront homeowner associations with 120 and 125 members, respectively. The Edgartown Harbor Association, the Friends of Sengekontacket (FOS), and Tisbury Waterways, Inc. (TWI) have larger memberships (200–450) that include contributors without pond frontage. Most of these organizations have tax exempt 501(c)(3) status, which encourages tax-deductible memberships.

The Tisbury Great Pond Think Tank, Edgartown Ponds District Advisory Committee, and Squibnocket Pond District Advisory Committee are more accurately described as agencies of local government, but all have significant neighborhood representation. Boldwater Association, a landowners group, is active in the Edgartown Ponds District, and the Riparian Landowners of Tisbury Great Pond participate in the Think Tank. Local government mandates the pond district advisory committees; the Think Tank is an ad hoc committee.

The regular annual budgets of these organizations are between $4000 and $25,000, which is usually raised through memberships, fundraising projects, and grants. Special projects have solicited much greater funding. The Lagoon Pond Association raised $75,000 to support the legal defense of its stand to limit construction of private docks. The Edgartown Harbor Association raised $750,000 in private donations to fund a Woods Hole Oceanographic Institution (WHOI) water quality study, which included a floating laboratory and scientific equipment. The Friends of Sengekontacket likewise has funded (over $90,000) studies by WHOI.

The effectiveness of these organizations is largely a function of their dedication. Environmental managers who have forged partnerships with these organizations have been rewarded with the energy, commitment, and passion that are reserved for issues "close to home." Waterfront land values are dependent on good

water quality, and local pond associations are the eyes and ears that sound the first alerts of environmental pollution. With the efficiency of local Minutemen, pond associations rally to the cause with volunteer environmental advocates who provide financial commitment, a wealth of expertise, and effective political organization. Most of the important shellfish ponds on Martha's Vineyard have shared jurisdiction between neighboring towns. The pond groups have provided valuable forums to coordinate the efforts of the boards of the adjacent towns, resulting in uniform management policies for the ponds.

The environmental accomplishments of neighborhood groups on Martha's Vineyard are impressive. All of the groups have been effective in funding water quality studies in their respective water bodies. Funding for these studies has been secured through private donations, town appropriations, and state and foundation grants. The Chappaquiddick Island Association, the Edgartown Harbor Association, and the Friends of Sengekontacket have contracted with WHOI for extensive investigations into sources of fecal coliform contamination and baseline surveys of chemical parameters. The Friends of Sengekontacket has funded inquiries into the impacts of dredging and proposed wastewater treatment, and mapped the bathymetry of its pond. The Tisbury Great Pond Think Tank has initiated land use studies for its watershed. Groundwater studies in the vicinity of Edgartown Great Pond, Tisbury Great Pond, and Chappaquiddick Island have been prompted by the pond groups. The Lagoon Pond Association funds an annual water-quality-monitoring program and conducts a survey yearly of the number of boat moorings. The Massachusetts Division of Marine Fisheries has conducted finfish surveys in Edgartown and Squibnocket ponds after urging by pond groups.

Armed with data from these studies, the pond associations have implemented a multitude of corrective measures. They have pressured the respective boards of Health to inspect and replace failed septic systems. The Lagoon Pond Association has also encouraged removal of underground oil tanks. Both the Edgartown Harbor Association and Tisbury Waterways, Inc. have addressed boat-related pollution. Edgartown presently provides free pump-out of boat sewage at its wharf. Tisbury Waterways, Inc. was instrumental in launching the traveling barge *PU. E - II*, which pumped 10,000 gallons of sewage waste from boat head-tanks in its second year of operation. Tisbury Waterways, Inc. also funds a summertime assistant to the harbormaster. This assistant patrols Lake Tashmoo distributing pamphlets and educating boaters about proper disposal of head tank waste.

The Lagoon Pond Association has proven itself to be an effective local political force. It was successful in its efforts to have the pond and its near shoreline declared a District of Critical Planning Concern and was instrumental in the establishment of pier guidelines to protect shellfish and eelgrass habitats.

Runoff from point sources and nonpoint sources has been addressed by a number of the organizations. The Think Tank mapped all road drainages into the shellfishing pond, and designed and implemented measures to redirect the road effluent into adjacent wetlands for filtration. The Think Tank worked with waterside farmers to fence livestock away from the shoreline, to adopt best management practices for handling manure, and encouraged the use of vegetative buffers to reduce agricultural runoff. Tisbury Waterways, Inc. in a cooperative project with the Board of Health secured a state grant for $50,000 to install and monitor innovative limestone catch basins to remove oils and metals, and neutralize acid-rain runoff.

The Chilmark Ponds Association, the Friends of Sengekontacket, and Tisbury Waterways, Inc. have coordinated and expedited dredging projects to remove sediments and improve circulation. Both Chilmark Ponds and the Friends of Sengekontacket, through its Barrier Beach Task Force, have developed management programs for the beaches and dunes adjacent to their ponds.

Public education is a high priority for the local associations. Through newsletters and annual reports, the membership are kept informed of ongoing projects and are provided with steps that they can take to ensure good water quality. The Edgartown Ponds Area Advisory Committee newsletter has a regular feature called Pond Reminders, which informs readers, among other things, that boats with antifouling paint are not allowed and that by law, a 100 ft no-cut buffer of natural vegetation must be maintained along the shore. The Think Tank produced and distributed a brochure with similar buffer recommendations, including a suggested list of native vegetation for landscaping. Both the Friends of Sengekontacket and Tisbury Waterways, Inc. have provided support for water quality education programs in the local schools. The Friends of Sengekontacket sponsors an annual "Carry In-Carry Out" anti-litter poster-contest for students, and the winning designs are posted on the island's ferries and at beach entrances.

In conclusion, neighborhood associations are established and potent forces in the preservation of shellfish habitats on Martha's Vineyard. They are models for effective stewardship of shellfish growing waters and should be duplicated elsewhere.

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MAKING A CASE FOR COMMUNITY-BASED OYSTER RESTORATION: AN EXAMPLE FROM HAMPTON ROADS, VIRGINIA, U.S.A.

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ABSTRACT The eastern oyster (*Crassostrea virginica*) remains at historically low levels throughout the Chesapeake Bay. Recent efforts to restore oysters in the bay have focused on establishing a series of sanctuaries, or no-take zones, to increase oyster broodstock in selected tributaries. Oyster parasites continue to affect the rate of recovery in these tributaries; however, innovative management strategies, advances in aquaculture technology, and the availability of disease-tolerant broodstock from the lower Chesapeake Bay are providing ways to involve the public directly in restoration of this resource. A 1996 management decision to transplant large wild-caught oysters onto an oyster broodstock sanctuary reef in the Great Wicomico River, Virginia, was followed by greatly increased abundance of juvenile oysters throughout that river in 1997. Using that result as a model for strategic oyster reef restoration, citizens and school students have been enlisted to grow large numbers of hatchery-produced native oysters for restocking other sanctuary reefs throughout Chesapeake Bay. Efforts to supplement natural oyster populations in Hampton Roads, Virginia, began in May 1998, with the transplanting of 65,000 hatchery-produced oysters grown by school students. The oysters were transplanted onto strategically located sanctuary reefs constructed in the Lynnhaven and Elizabeth rivers. Surveys of these reefs following the oysters' spawning season have revealed order-of-magnitude increases in the abundance of juvenile oysters on both reefs, and correspondingly high spat settlement rates on oyster grounds surrounding the reefs. These results demonstrate that stocking strategically located broodstock reefs with hatchery-produced oysters grown by citizens can be an effective strategy for oyster restoration in the Chesapeake Bay.

KEY WORDS: *Crassostrea virginica*, oyster, habitat, restoration, fisheries management

INTRODUCTION

The tremendous decline in the abundance of the eastern oyster, *Crassostrea virginica*, in the Chesapeake Bay is attributed to several factors: excessive harvest pressure in the late 1880s and early 1900s, declining water quality and increasing sedimentation rates, and more recently, the presence of two disease-causing oyster parasites commonly known as MSX and Dermo (Kennedy and Breisch 1983; Hargis and Haven 1988, 1995; Ford and Tripp 1996). As a result, oyster landings have plummeted in Virginia to less than 1% of the levels in the mid 1900s (Fig. 1). As recently as the 1980s, the oyster fishery was the most valuable commercial fishery in the bay. At present, however, Virginia's oyster fishery supports the equivalent of a mere seven full-time jobs, and the region's oyster-processing industry relies on the importation of oysters from outside state waters to support public demand for this resource (Kirkley 1997).

In addition to the economic value that might be realized from a restored oyster fishery, a broad-based effort to restore the Chesapeake's oyster populations could also yield profound ecological benefits. The role of oysters as a dominant suspension-feeder is well documented. For example, Newell (1988) estimated that historic (pre-1870) oyster populations were capable of processing significant fractions of the bay's water volume each day. Recent studies suggest that restoring oyster populations to the Chesapeake Bay, particularly along the shallow margins, could significantly reduce concentrations of suspended particulates, improve light penetration, and increase dissolved oxygen concentrations in the bottom waters (Newell 1988; Ulanowicz and Tuttle 1992; Gerritsen et al. 1994; Kennedy 1996). Indeed, the concept of restoring water quality through revitalized oyster stocks is increasingly understood and accepted by the public, as evidenced by a recent article in U.S. News & World Report (Zimmerman 1997) that described oyster restoration in the Chesapeake Bay as one of "Sixteen Smart Ideas to Save the World."

Recent efforts to restore oysters to Virginia's tributaries have two primary strategies: construction of oyster habitat using large volumes of shell to recreate three-dimensional reefs on historic oyster grounds, and management of the remnant fishery to increase the abundance and size of oyster broodstock on public oyster grounds. A system of reconstructed reefs designated as oyster broodstock sanctuaries (no-take zones) have been established in Virginia tributaries (Fig. 2). The sanctuary reefs are intended to allow oysters to accumulate, mature, and reproduce, thereby enhancing local oyster populations. An experiment in the Great Wicomico River in 1996 used large adult oysters purchased from oyster fishermen to stock one such reef. The experiment resulted in a significant increase in oyster settlement onto the sanctuary reef

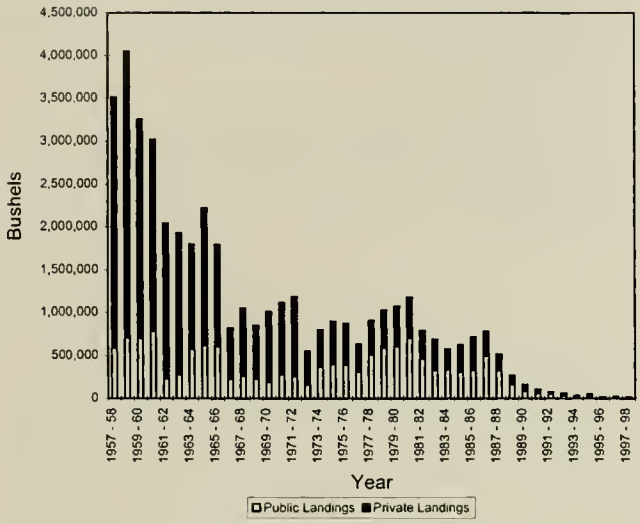

Figure 1. Oyster landings in Virginia between 1957 and 1998. Landings are divided between private and public oyster grounds.

and surrounding oyster grounds the following year (Fig. 3A,B) (Southworth and Mann 1998). Using this result as a model for oyster restoration in other tributaries, a program involving private citizens and school students in the grow-out of hatchery-produced oysters for stocking sanctuary reefs was implemented in Hampton Roads, Virginia, in the lower Chesapeake Bay. In the first year of this effort, approximately 90,000 hatchery-produced oysters grown by citizens and students were transplanted to reefs located in the Lynnhaven and Elizabeth rivers.

Data collected by students participating in the Chesapeake Bay Foundation's (CBF) Student Oyster Corps, along with surveys of the sanctuary reefs after the transplanted oysters had spawned, were used to evaluate the success of this project. The results demonstrate that a citizen-based restoration effort using strategically located sanctuary reefs and hatchery-produced oysters can lead to significant increases in local oyster stocks.

METHODS

Large, presumably disease-tolerant oysters were collected from the Lynnhaven River in spring of 1997 and transported to a commercial hatchery for spawning (Middle Peninsula Aquaculture, North, Virginia). Oyster larvae were settled onto shell grit to produce "cultch-less," or individual, juvenile oysters. The broodstock oysters were spawned in the hatchery on June 5, 1997, and juvenile oysters with a mean size of 26.2 mm were distributed to school classes on October 4, 1997.

Teachers and students from 26 middle school and high school classes grew the hatchery-produced oysters in floating cages secured to docks in tidal waters near each school. Students from each class constructed floating cages measuring 8 × 2 × 1ft (244 × 61 × 30 cm) using sewer-grade PVC pipe and vinyl-coated wire mesh (14-gauge 25-mm square mesh). Each cage contained 2000 oysters divided between two large plastic mesh bags (mesh size 5 mm). The cages were placed in the water at a dock or marina convenient to each class's school and were monitored monthly. When appropriate, oysters were transferred to bags with larger mesh sizes to minimize restriction of water flow.

Students monitored oyster growth and survival and measured surface water temperature, salinity, and turbidity; monthly data sheets were submitted to CBF throughout the school year. Random samples of 40 oysters were taken from each floating cage (20 from each of the two mesh bags) and measured to the nearest millimeter. Each class was provided with a refractometer (SPER Scientific model A366ATC) and was instructed to calibrate the instrument with distilled water prior to use each month. Secchi disks (20 cm diameter) were provided for measuring water clarity.

At the end of the school year, the students transplanted their oysters to sanctuary oyster reefs located in the Lynnhaven and Elizabeth rivers in the lower Chesapeake Bay. The oysters were transplanted to the reefs in high densities (approximately 200–300/m^2) just below the MLW level in an effort to increase fertilization success upon spawning. The reefs in the Lynnhaven and Elizabeth rivers were constructed in May 1997 and May 1998, respectively, using barge loads of clean oyster shells deposited on the river bottom. Both reefs are approximately one-half acre in size and rise 1–2 m above the bottom, extending to approximately 0.25 m above the MLW level.

Data submitted by the school classes was pooled across sites to obtain monthly averages for oyster shell length, salinity, temperature, and water clarity. Monthly growth rates were computed and correlated to water quality parameters. After the transplanted oysters had spawned, recently settled juveniles (spat) were surveyed

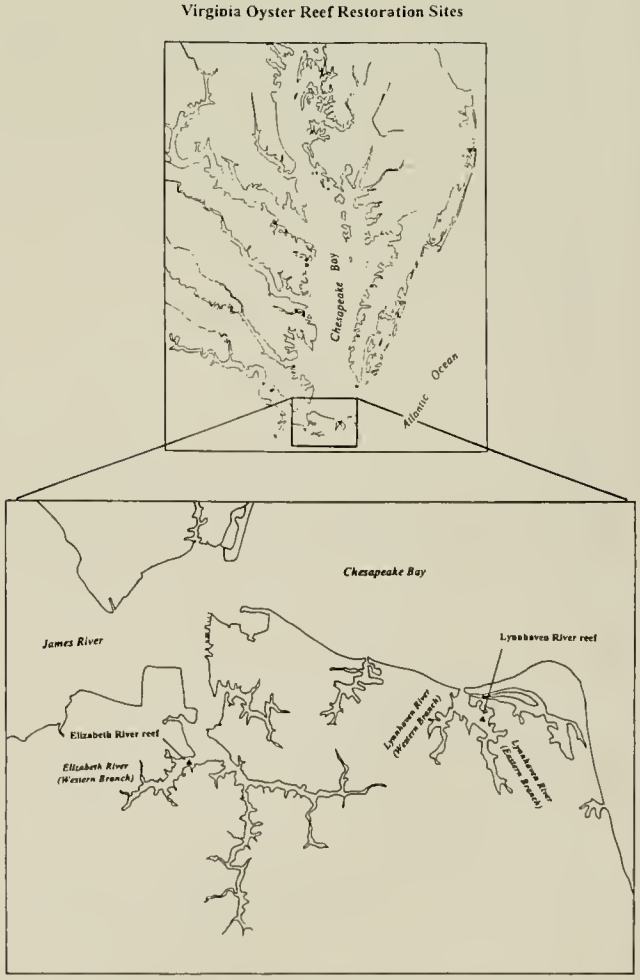

Figure 2. Locations of sanctuary reefs in Virginia's portion of Chesapeake Bay. Inset shows locations of sanctuary reefs in the Lynnhaven and Elizabeth rivers in Hampton Roads, Virginia.

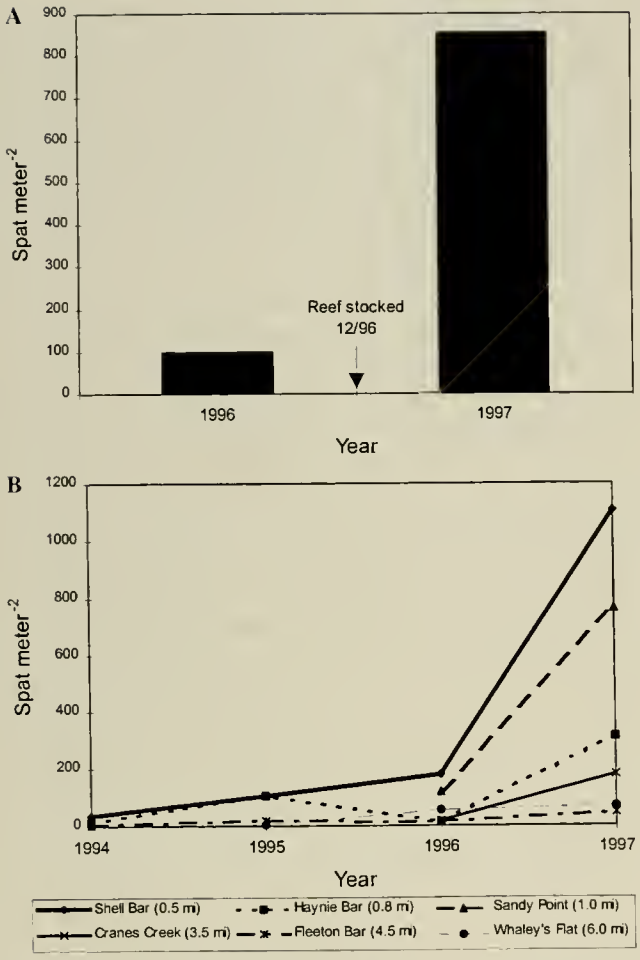

Figure 3. (A) Mean abundance of juvenile oysters (spat/m^2) on the Great Wicomico River sanctuary reef. (B) Mean abundance of juvenile oysters (spat/m^2) on public oyster grounds in the Great Wicomico River. Symbols represent different natural oyster bars near the reef. Distance from the reef is in parentheses.

on both reefs in October 1998 using 0.25-m^2 quadrats. Twelve replicate samples were obtained (using SCUBA) from each reef, with samples divided evenly among high, middle, and low reef elevations. Additional samples were taken from oyster grounds throughout the Lynnhaven River that were exposed at low tide. These sample were used to determine how far from the reef oyster spat might have been transported prior to settlement.

RESULTS

Student-grown oysters increased from 26.2 mm in October 1997 to approximately 50 mm by June 1998, when the oysters were transplanted to sanctuary reefs. A clear seasonal cycle of growth was evident, with minimal growth observed between December 1997 and April 1998 (Fig. 4A). Juvenile mortality was less than 1% throughout the school year. One hundred oysters from the same cohort were retained in floating cages and monitored for growth and survival throughout 1998. By October 1998, these oysters had increased to 77 mm, and mortality was only 6%.

Both temperature and salinity exhibited seasonal cycles within documented ranges that favor oyster growth and survival (Fig. 4B) (Galtsoff 1964; Loosanoff 1953, 1958). Temperature varied from 11 °C in the winter to 27 °C in July 1998. Mean salinity reached a maximum of 25 ppt in October 1997, and a minimum of 10 ppt in February 1998 following several winter "Nor'easter" storms that brought heavy precipitation to the region between December and February. Salinities remained low through the spring as a result of higher than average precipitation rates in the region. Water clarity (Secchi depth) showed two maxima in December and March and ranged from less than 1 m to more than 2 m throughout the year. The peak in December most likely reflects the decline in phytoplankton biomass following the fall bloom, while the decrease through February was most likely associated with heavy runoff and suspended solids from winter storm events.

A strong correlation was observed between salinity and growth rate ($r = 0.86$, $P < .003$). However, there was no apparent correlation between temperature and growth, or between water clarity and oyster growth. Spat settlement increased dramatically on the Lynnhaven River sanctuary reef between 1997 (prior to stocking) and 1998 (post-stocking), from 8 to 181 spat/m^2 (Fig. 5A). While similar pre-stocking data are not available for the Elizabeth River reef, spat settlement there in 1998 was similar in magnitude to the Lynnhaven reef. In addition, dredge samples taken from shell bottom close to the Elizabeth River reef suggest the observed settlement rates represent a substantial increase over previous years.

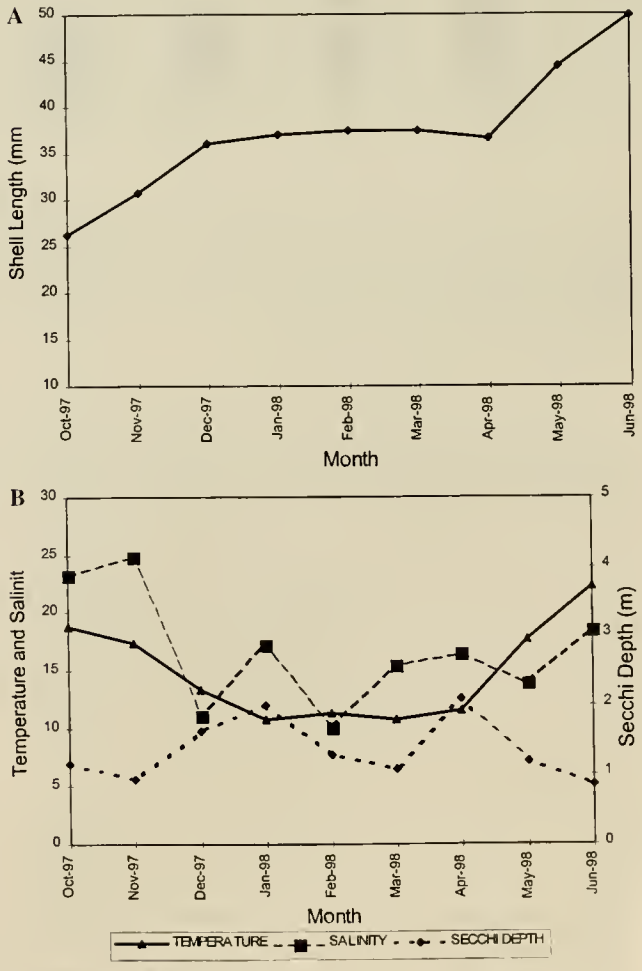

Figure 4. (A) Mean size of hatchery-produced oysters grown by students. (B) Mean monthly surface water temperature, salinity, and water clarity.

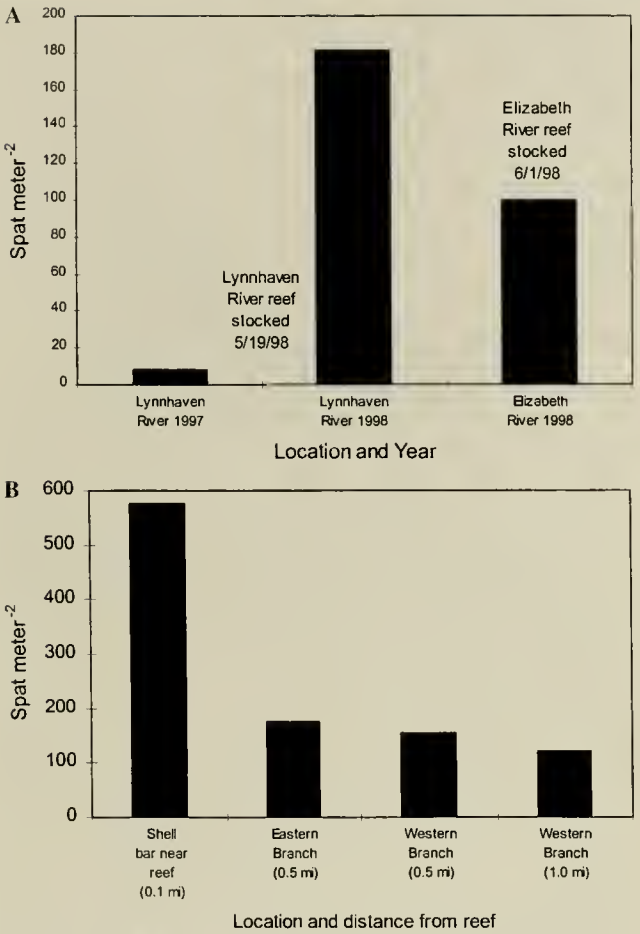

Figure 5. (A) Mean abundance of juvenile oysters (spat/m^2) on Hampton Roads sanctuary reefs. (B) Mean abundance of juvenile oysters (spat/m^2) on intertidal oyster grounds in the Lynnhaven River in 1998. Distance from the reef is in parentheses.

DISCUSSION

The results of this first year of effort demonstrate that stocking even modest numbers of hatchery-produced oysters derived from hardy broodstock is useful for restoration of the Chesapeake Bay's oyster populations. Spawning by oysters from the same cohort held in floating cages through the summer was observed on July 10, 1998 (pers. obs.), which supports the notion that the transplanted oysters are capable of spawning after being transplanted to the reefs. Dredge samples taken near the Elizabeth River reef revealed that spat were 10–20 times more abundant than juvenile and adult oysters, suggesting poor recruitment prior to 1998. Moreover, there are few "boxes," or empty shells, which indicates that recent oyster mortality is relatively low. Therefore, settlement appears to be a limiting factor for oyster populations in this river.

Similarly, spat were frequently the only live oysters found in quadrat samples from unrestored shell bottom in the Lynnhaven River. Spat abundance on natural shell bottom was highest near the reef and decreased with distance from the Lynnhaven reef (Fig. 5B), similar to the trend observed in the Great Wicomico River in 1997 (Fig. 3B).

While the absolute abundance of spat differs between the Great Wicomico River and Lynnhaven River reefs (856 versus 181 spat/m^2), the order-of-magnitude increases in spat settlement observed in both systems following initial stocking efforts are strikingly similar. It is worth noting that in places like the Great Wicomico River, where "background" spat settlement in recent years has been on the order of 100 spat/m^2, the impact of stocking efforts as modest as those conducted in Hampton Roads in 1998 may not be detectable. However, in places like the Lynnhaven and Elizabeth rivers where spat settlement rates in recent years have been considerably lower (e.g., 8 spat/m^2 in the Lynnhaven in 1997), these effects are more easily discerned.

The data collected in 1997 and 1998 by the student volunteers demonstrate that, even in highly urbanized areas, restoration efforts using hatchery-produced oysters and small-scale aquaculture techniques can be effective. Juvenile mortality was very low throughout the school year, and temperature and salinity ranges were suitable for oysters to grow to maturity by mid-summer after their first year of growth. Although the effect of suspended particulate matter on oyster feeding rates is well documented (e.g., Loosanoff 1962; Loosanoff and Tommers 1948), there did not appear to be any relationship between water clarity and oyster growth in this study. It is possible that suspended sediment levels never exceeded threshold levels that decrease feeding rates, or that the particulate material in the water column was not of an appropriate size fraction to affect the oysters' growth adversely. More likely, growth was the result of synergistic effects between two or more of the water quality parameters measured by the students (Kennedy 1991). These relationships will be examined more closely as more data are obtained through this program in subsequent years.

The Lynnhaven River is located in the high-salinity portion of lower Chesapeake Bay where diseases have caused extensive oyster mortality in recent decades (Burreson and Calvo 1996, and references therein). Large, isolated oysters that survive in such areas are thought to be more tolerant of the parasites MSX and Dermo than smaller oysters (Gaffney and Bushek 1996) and thus, are deemed more valuable as broodstock. While the ability of transplanted oysters to pass on the trait of disease-tolerance is poorly understood, concentrating large, presumably disease-tolerant oysters on these reefs is thought to offer some hope of overcoming the reproductive failure resulting from low densities of broodstock. Surveys of the reefs and surrounding bottom in future years will better illustrate the effect of broodstock selection on restoration efforts.

Our strategy of using hatchery-produced shellfish as broodstock parallels efforts to restore shellfish stocks in other systems. For example, in the Westport River, Massachusetts, a community-based effort to restore bay scallops, *Argopecten irradians*, has been undertaken using hatchery-produced scallops and spat collector bags deployed in arrays throughout that system (Tammi et al. 1998; Turner and Soares 1998). In areas within that river having historically high settlement of scallops, hatchery-produced scallops were grown to maturity by volunteers and held in floating cages in an attempt to enhance scallop fertilization rates. Spat collectors, consisting of monofilament fishing net material, were manufactured by school students and citizen volunteers and used to collect juvenile scallops for further enhancement of natural stocks.

One of the less tangible, but nevertheless important, benefits of the Virginia oyster restoration effort is the increased public awareness that has followed. Funding for reef restoration efforts typi-

cally has been through grants to the Virginia Marine Resources Commission (VMRC) from outside sources, such as the U.S. Environmental Protection Agency (USEPA), National Oceanic and Atmospheric Administration (NOAA), and Virginia's Chesapeake Bay Restoration Fund (whose monies are derived from the sale of "Friend of the Bay" license plates). As a result of these early restoration successes additional private sources of funding are being made available for restoration work. For example, the Rotary Club of Norfolk, Virginia, committed $28,000 toward construction of two additional reefs in Hampton Roads, which were constructed in the Lafayette River in 1999.

In many parts of Hampton Roads, water quality conditions prevent or restrict the taking of shellfish for human consumption. Polluted waters are classified as either restricted, whereby shellfish must be relayed according to specific state guidelines, or prohibited, in which case no shellfish may be removed for consumption. Despite these conditions, there is increasing public support for restoring oyster reefs strictly for their ecological potential, such as increased water clarity and the provision of fish habitat. The public perception of oysters as a keystone species in the bay, combined with existing support for the continuation of fishery enhancement efforts, is especially encouraging. It demonstrates public commitment to programs designed to restore the Chesapeake Bay, and an increasing appreciation for the ecological, as much as economic, importance of natural resources.

Since the initiation of this restoration project, CBF's Student Oyster Corps has expanded to include more than 90 classes baywide. A local citizens group called Restore the Oyster formed in 1997 to help advance oyster restoration efforts in Hampton Roads by recruiting citizens to grow oysters for stocking local reefs. More than 250,000 hatchery-produced oysters reared in floating cages by citizens and students in CBF's Oyster Corps program were transplanted to the reefs in 1999. Additionally, selectively bred oysters have been made available to commercial hatcheries and are now being used to produce seed oysters for this effort. The first of those oysters were transplanted to reefs in the spring of 1999. Continued monitoring of the sanctuary reefs and surrounding oyster grounds will better define the impacts that these stock-enhancement activities are having on local oyster populations.

ACKNOWLEDGMENTS

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JUMP RUN CREEK SHELLFISH RESTORATION PROJECT

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ABSTRACT The objective of this multi-agency project is to 1) quantify the effects of land use change on shellfish closures and 2) assess techniques that can be used mitigate those impacts. This report is based on preliminary analyses conducted over the last 18 months. The project focuses on the 320-ha watershed in Carteret County, North Carolina, that is the drainage for Jump Run Creek. Bacterial data from 1970 through 1998 indicate increased loading since 1974, which is when closure management began. Recent grab sample data from the tributaries indicate high levels of bacteria during storm events and moderate levels during dry weather. The majority of the loading is coming from the portion of the watershed draining an older, medium density neighborhood (single family houses) and a trailer park. A door-to-door survey found two malfunctioning septic systems, more than 100 pets, and the presence of wildlife. Change analysis of land use/land cover shows hydrologic modifications were instituted in the 1970s. Dye studies confirm that water moves through the watershed in hours, indicating that time for bacterial mortality is insignificant. Future analyses include ground water sampling, automated storm water monitoring, and DNA tracking of fecal sources. Planned mitigation practices will include riparian buffer restoration, stormwater wetland, bio-retention, peat filters, and education. Locations and sizing of practices will be determined through GIS-based hydrologic analysis of the watershed in conjunction with a community-design/educational approach involving neighborhood citizens.

INTRODUCTION

This project focuses on restoration of a watershed draining to a shellfish resource at the mouth of Jumping Run Creek. The 320-ha watershed contains mixed land use including single-family residential, business, and industrial facilities. The natural land cover is dense, coastal pocosin growing above sandy, relic dune ridges. In the upland portion of the creek, both banks are heavily forested with wide riparian buffers. The lower portion of the creek is bordered by residential lawns with banks that are bulkheaded.

The creek empties into a significant shellfish resource for which closure management began in 1974. The areal extent and length of closure has increased steadily so that now the entire resource is permanently closed. This is the trend for shellfish resources throughout North Carolina's coastal region, with a 12% increase in closures in the last 5 years.

Increased closures have occurred simultaneously with increases in population. However, like Jumping Run Creek, closure management is occurring in areas impacted by creeks draining watersheds with extremely low development densities. This fact concerns local residents, fishing interests, Shellfish Sanitation staff, and marine researchers. The objective of this project is to investigate why this is happening and how it can be alleviated by 1) quantifying the sources and loading rate of bacterial contamination and 2) by testing watershed-based restoration techniques for mitigation.

METHODS

Site Description

There is a variety of land uses within Jumping Run Creek watershed (Fig. 1). Twenty-four hectares are mobile (trailer) home and recreational vehicle (RV) housing. This area is characterized by gravel roads, grassed drainage swales, large trees, and lawns. The trailer park uses traditional septic systems for waste manage-

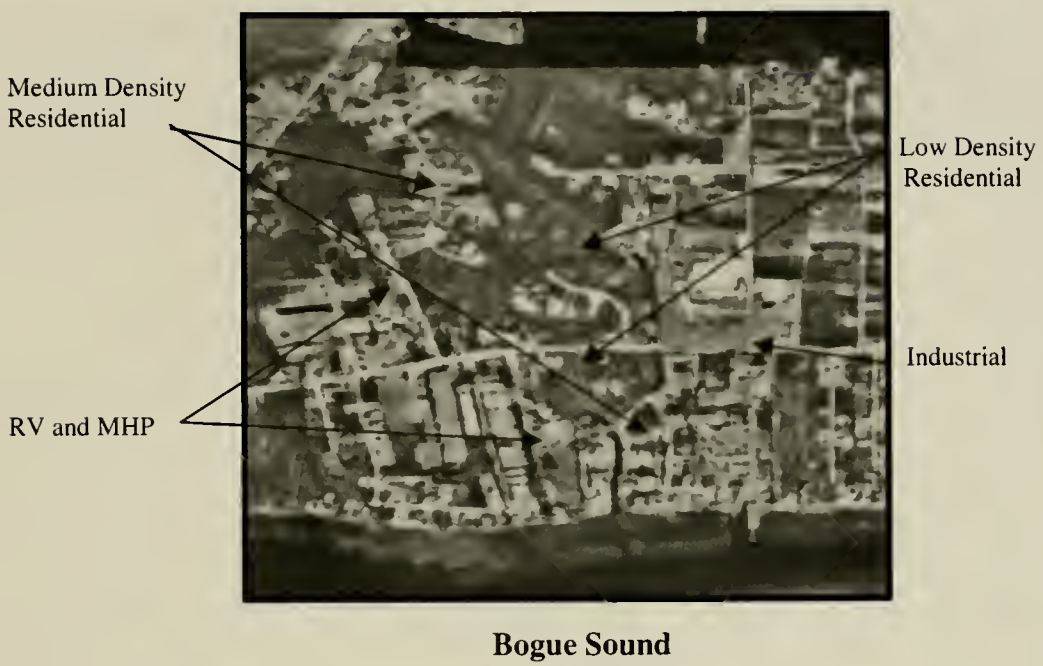

Figure 1. United States Geological Survey 1994 aerial photograph with noted land use.

ment. The RV park has a new, low-pressure pipe septage treatment (LPP) system. Low-density, single family residential (greater than 0.40-ha lots) encompasses 48 ha. Roads and some drives are paved, but there are no sidewalks, and stormwater drainage is conveyed in grassed swales. The lots are characterized by large trees, expansive lawns, with wooded buffers between houses. Waste management is traditional septic systems. Medium-density residential (lots less than 0.4 ha) covers 109 ha. In the upper part of the watershed, roads are paved, drives are gravel, and stormwater conveyance is in grassed swales. Lots are covered primarily by lawn and large trees and the creek area has a tree-based riparian zone. In the lower portion of the watershed, drives and roads are paved with both piped and grassed swale stormwater conveyances, bulkheaded yards, and cleared creek edges. Waste management in both areas is traditional septic. In the industrial zone, which covers 93 ha, there are paved roads and parking lots, sidewalks, gutters, and combinations of grassed swales and piped stormwater conveyances. Buildings have small footprints and expansive lawn areas, ornamental trees, and shrubbery beds. Waste management is in-ground septic systems. Open space encompasses 48 ha of undevelopable steep slopes and pocosins along creek banks. These landscape features are indicative of the White Oak River system, which is composed of relic dunes ridges, steep banks, and heavy vegetation.

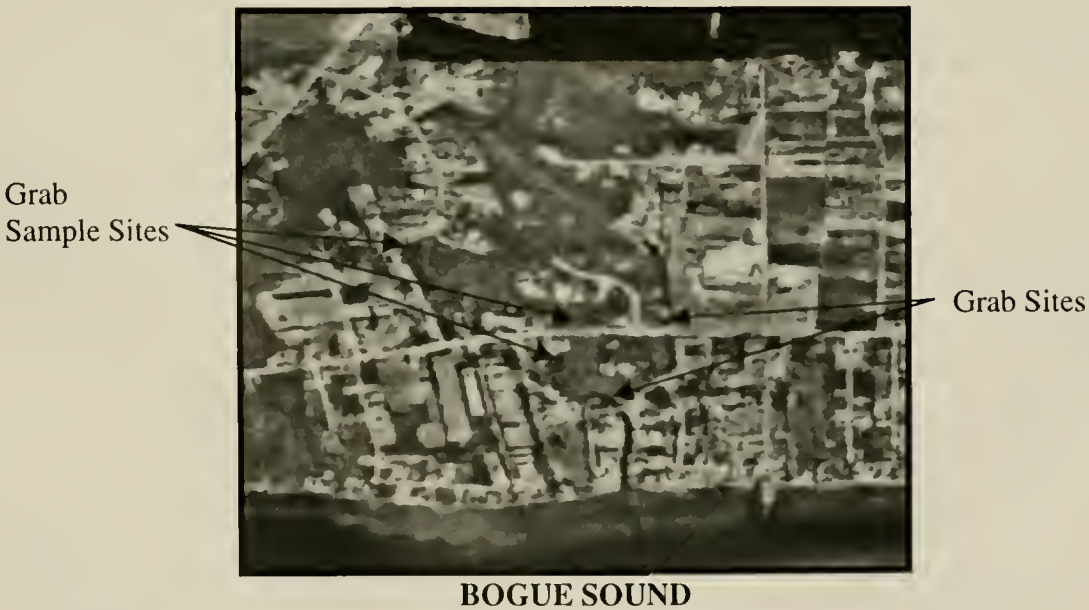

Figure 2. United States Geological Survey 1994 aerial photograph on which sample locations are identified.

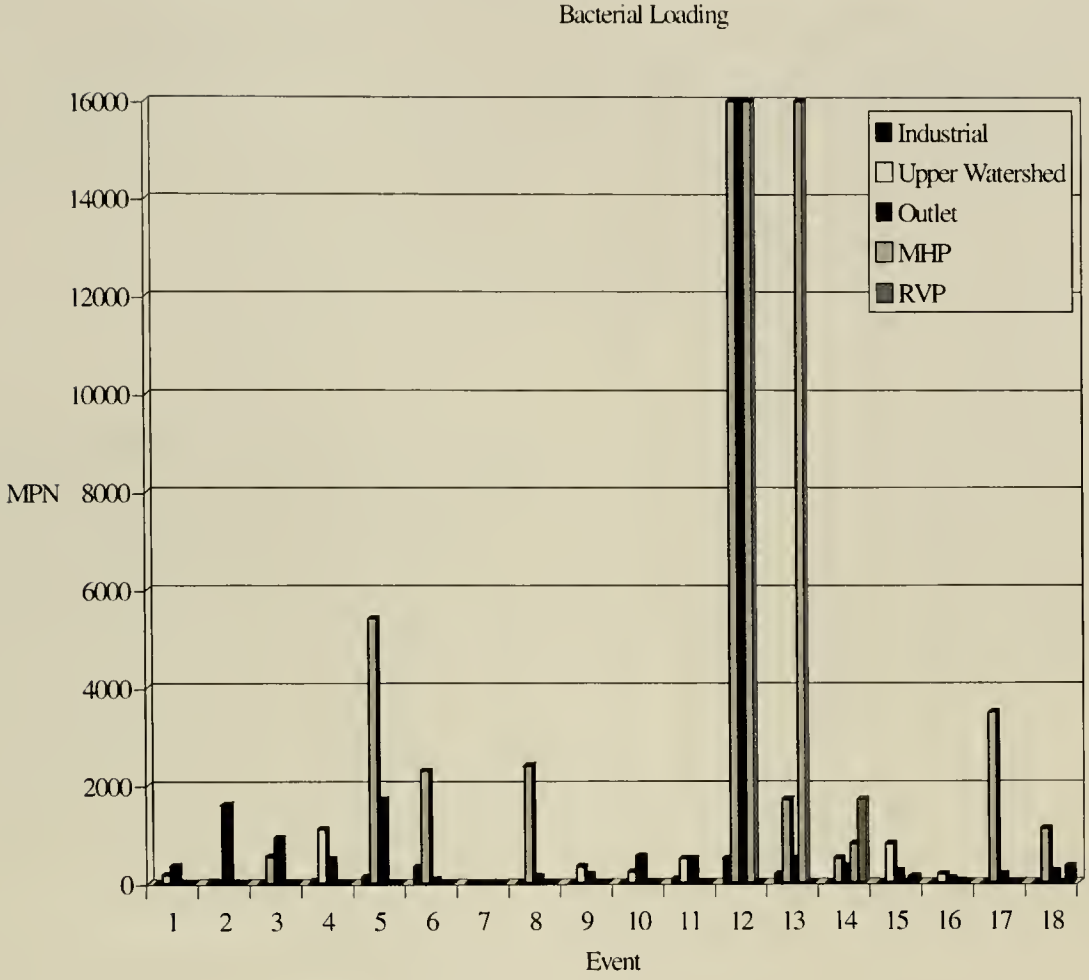

Figure 3. Results of bacterial grab samples by location.

Sampling

Grab samples were collected after storm events and analyzed for fecal coliform, nutrients, and sediment. Fecal coliform analysis was conducted by Shellfish Sanitation of the North Carolina Department of Environment and Natural Resources (NC DENR) in order for project work to be consistent with their data. The sample sites were located to characterize the water draining from trailer park, residential areas, industrial facilities, RV park, and at the outlet of the creek before it is influenced by tidal waters (Fig. 2). Samples from the creek outlet were collected at the lowest point of the ebb tide.

Rhodomine dye studies were conducted to determine the time needed for water flowing from each portion of the watershed to reach the grab sample sites. The flow rates are collaborated with data collected using a propeller-type, velocity flowmeter. Water level relative to flow was noted using an in-stream gage plate. The dye was dripped into the creek at a measured concentration and rate using a peristaltic pump. Time was kept until the centroid of the dye, as determined using a fluorometer, reached its destination. This information and the flowmeter data were used to calculate velocity in cubic meters per second (CMS).

The day after a 254-mm rainfall event, the research team conducted a door-to-door survey in the watershed. Each survey team included a certified Health Inspector as well as member of the research team. At each location, septic leach fields were located using a metal probe and checked for inundation as well as surfacing septage. The imperviousness of the sites was measured. In addition, residents were queried as to the number of pets in the household.

RESULTS

The results of the bacterial sampling are summarized in Figure 3. After 11 sampling events, the majority of the loading was determined to be coming from the upper reaches of the watershed containing the trailer park and residential land uses. Additional samples were collected at the tributary below the trailer park and RV park to examine this in greater detail. These data indicated that very little additional loading occurred as the water flowed past the RV park to the outlet. Contributions from the industrial area were inconsequential and grab sampling at this location was suspended. Data show levels from the trailer park, creek outlet, and below the upper reaches of the watershed are similar, indicating that for these events the trailer park area was contributing the majority of the bacteria to the water column.

The results of the dye studies are summarized in Table 1. It is important to note that at relatively low flow levels, water moves through the system in less a than a day. Furthermore, during winter months when flow levels are high, bacterial life is extended (White

TABLE 1.

Summary of travel time.

Date (1998)	Gauge Hgt (m)	CMS	Travel Time
January 21	0.42	0.23	No Data
January 22	0.35	0.16	MHP-Outlet, 5 hours
February 4	0.91	0.50	MHP-Gauge, 2.5 hours
February 23	0.51	0.30	No Data
March 3	0.34	0.08	Headwaters-Gauge, 3 hours
March 4	0.33	0.07	RVP to Outlet, 1.5 hours
April 3	0.28	0.16	No Data
April 17	0.27	0.29	No Data
April 30	0.25	0.14	No Data
June 18	0.23	0.12	No Data
July 14	0.21	0.12	No Data

TABLE 2.

Summary of neighborhood survey.

Location	Visits	SV	Dogs	Cats	Other Pets	Imperviousness
MHP	47	0	23	6	3	3716 m^2
Medium Density	66	2	60	47	25	17,861 m^2
Low Density	14	0	7	0	0	8740 m^2
RVP	1	0	0	0	0	460 m^2
Totals	128	2	90	53	28	29,900 m^2
						2.9 ha

* Does not include roads or industrial area.

1996) due to low temperatures and reduced light levels, and the contamination potential is higher.

Neighborhood survey results are summarized in Table 2. There were only two septic system surface failures, less than 10% imperviousness, and more than 30 cats at one location—most pets were located close to tributaries.

CONCLUSIONS

The surface septic system failures, found during the survey, were not connected to the creek via surface runoff. Hence, these did not appear to be a source of bacterial contamination. However, the possibility that bacteria are transported via groundwater has not been dismissed. The research team is concerned that septic leach fields may be intersecting groundwater during wet weather, and, in combination with porous, sandy soils, leaching bacteria into the creek.

Lack of bacterial presence in grab samples eliminated the industrial area from consideration as a contributor. However, this area is contributing large volumes of fresh stormwater, which is a problem. NC DENR Wetland Restoration Program is participating in the project, and they, based on project data, are planning to convert some of the lawn areas, which are being donated by the landowner, to wetlands. This will increase storage time and filtration as well as reduce the flow volume and velocities during storm events of the water draining from more than one fourth of the watershed.

Even counting the roads and industrial park areas, this watershed is less that 5% impervious, which is well below the published threshold for the initiation of water quality degradation (Schuler 1995). However, preliminary examination of aerial photography indicates that extensive hydrologic modification of all land cover has occurred. Photographs from the 1930s through the 1990s show channelization, ditching, and bulkheading—modifications that cause stormwater runoff to be delivered faster and in greater volumes during storm events, allowing less time for bacterial stores in the watershed, naturally occurring or not, to be reduced. These photos will be further analyzed using GIS-based, land use/land cover, time-change analysis to quantify where the most significant changes have occurred. These data will be analyzed spatially relative to 30 years of bacterial data for potential correlation.

Nevertheless, the impact of such alterations on bacterial loading potential are not captured adequately by measuring impervious surfaces. The hydrologic transport mechanisms as well as the location of bacteria needs to be known in order to properly target and design mitigation. To further this end, automatic monitoring stations have been installed in the watershed below the trailer park, at the gage, and near the creek outlet. These will collect flow-weighted storm samples, which will be composited for the rising, peak, and falling portions of the storm hydrograph. These data will clarify when the bacteria is loading, and in conjunction with base-flow-oriented grab samples, assist in differentiation between transport pathways and the calculation of total loading by land use. If necessary to determine source and transport path, dye studies will be conducted on septic systems in the watershed and DNA analyses on the fecal matter.

Once loading and transport pathways are known, the project team plans to design and install innovative elevated bioretention areas, peat and sand filters, and constructed wetlands to mitigate the bacterial loading. In 1999, a neighborhood stakeholder group participated in a design charette to assist in this effort. In this manner, these facilities become neighborhood amenities not just stormwater treatment devices.

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ONE SHINING MOMENT KNOWN AS CLAMELOT: THE CEDAR KEY STORY*

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ABSTRACT The heritage and culture of rural communities along Florida's Big Bend coastline in the Gulf of Mexico have been linked intrinsically with commercial fisheries for generations. Over the past decade, closures of oyster harvesting grounds and a state-imposed ban on gill nets triggered economic decline and depression in this area. A transition to shellfish aquaculture as an alternative employment opportunity has been facilitated through the recent federally funded, job-retraining programs. Since 1993 when the first program graduates were placed with leases, the industry has grown and now supports over 300 hard clam, *Mercenaria mercenaria* (Linnaeus, 1758), growout operations on 950 acres of state-owned submerged lands with sales (farm gate value) estimated at $10 million in 1997. The promise of prosperity has created a new excitement and common bond among the individuals of these communities. With a renewed sense of purpose and cohesion, people are working together to promote their livelihoods, and above all, to protect the coastal waters so critical to the success of these ventures. This revitalization has also spurred a reaction and responsiveness to the emergent industry by local governments and by state and federal agencies. Citizens of Cedar Key have formed advisory groups to work closely with elected officials and agency representatives in the planning and implementation of a wide range of water quality activities. These include storm water and wastewater treatment, environmental education, and water quality monitoring programs. Sustainable hard-clam aquaculture operations have proven to be an excellent opportunity to both protect and preserve the region's environmental qualities as well as support economic activity.

KEY WORDS: *Mercenaria mercenaria*, hard clam, aquaculture, partnership, pollution abatement, shellfish restoration and remediation, water quality monitoring, watershed management

IN THE BEGINNING

*"Ask every person if he has heard the story and tell it strong and clear if he has not, that once there was a fleeting wisp of glory called Clamelot."**

The history, character, heritage, and economy of this modern-day *Clamelot* are inextricably tied to Cedar Key's scenic, teeming coastal waters. Tourism, annual seafood festivals, restaurants, and commercial and recreational fishing all depend on good quality water. Yet, during the past decade, broken septic and stormwater systems have led to broken dreams, as a large number of the area's commercial oystermen and fishermen were forced out of business by contaminated Gulf of Mexico waters. With a renewed sense of purpose and determination, the community is now working together to develop and promote new livelihoods and to protect the coastal waters so vital to the success of these commercial ventures and the survival of their beloved *Clamelot*.

CREATING NEW JOB OPPORTUNITIES

*"It's true, it's true the climate must be perfect, all the year. In short, there's simply not a more congenial spot for happy everaftering than here in Clamelot."**

Florida's warm Gulf waters and high natural productivity levels create a superb environment for marine life and, by extension, for those who earn their living "on the water." The weather, market trends, and a variety of other factors have always made life interesting, and at times uncertain, for local residents. Yet, the economic picture in *Clamelot* has not always been "rosy"—it has included the closure of oyster harvesting grounds due to water pollution and a state-imposed ban on gill nets.

Economic survival in *Clamelot* requires a willingness to adapt, and its residents have embraced a promising new industry with gusto. Beginning in 1991, the Florida Department of Labor and Employment Security introduced federally funded, job-retraining programs in shellfish aquaculture for unemployed or underemployed oyster harvesters and other seafood workers in a four-county area. Trainees were prepared for their new businesses through hands-on participation and a classroom curriculum. Instruction was provided by Harbor Branch Oceanographic Institution and the University of Florida's Institute of Food and Agricultural Sciences. The programs, Project OCEAN and Project WAVE, were headquartered in Cedar Key.

Through Project OCEAN, which incorporated both oyster and hard clam culture technology, over 130 program graduates received shellfish aquaculture leases in 1993 and the knowledge to put the submerged lands into production. The success of this program was the impetus for Project WAVE, which enabled displaced net fishermen in the same region to be instructed in the business of culturing hard clams. During 1995–1997, 69 fishermen were given leases for the startup of individual- or family-operated farms. Most of the trainees have made a successful transition to clam farming and are operating productive and profitable leases. Currently, the emergent industry now supports more than 300 hard clam growout operations on 950 acres of state-owned submerged lands off the coast of Dixie and Levy counties. Sales, (farm gate value) in 1997 were estimated at $10 million. Shellfish aquaculture is now a primary source of income for many residents along the coast.

*With apologies to Alan Jay Lerner and Frederick Lowe, whose wonderfully appropriate lyrics to the musical *Camelot*, published in 1960, were the inspiration for this presentation.

Figure 1. Clam farmer harvesting his crop from lease areas located in the productive waters of the Gulf of Mexico off Cedar Key, Florida.

COMMUNITY INVOLVEMENT

There may be only one road leading in and out of Cedar Key, but there are many paths leading to the protection of the town's water quality and, consequently, its lucrative shellfish industry. When a statewide gill net ban was imposed in 1995, many commercial net fishermen found themselves out of work. Looking to reverse their misfortunes, they turned to clam farming, a newly emerging industry in which success is directly tied to water quality from the estuaries and from human activities in and around Cedar Key.

When inadequate stormwater and sewage treatment systems began posing threats to local water quality, citizens rallied to protect the natural resources on which their livelihoods were dependent. They formed the Cedar Key Water Alliance to encourage citizen participation in finding solutions to some of the towns most pressing water resource concerns. The committee's advisory groups worked closely with elected officials and agency representatives in planning and implementing a wide range of water quality activities, including improved stormwater and wastewater treatment systems and environmental education. The community received substantial funding from the state's Surface Water Improvement and Management Program to conduct a master stormwater system study and to develop a master stormwater plan. An additional $500,000 has been appropriated for implementation of stormwater projects, with funds provided through the Florida Department of Transportation's wetlands mitigation program. One of Cedar Key's top priorities has been to replace all existing septic tanks with connections to the town's centralized sewer system. To achieve this ambitious goal, volunteers surveyed existing homes, as well as lots not yet on the system, and drafted a budget for both short-term and long-term goals. Homes that were within the existing collection area were targeted initially. With a $52,000 grant from the Suwannee River Water Management District to purchase the necessary supplies, committee members provided the labor to connect over 42 homes. Next the group sought to expand the sewer system to serve the more than 100 remaining homes still on septic tanks. The city and its water and sewerage district garnered support from their local legislative delegation, and in 1998 the Florida Legislature appropriated $790,000 to eliminate every septic tank in the community by the year 2000. The result will be ongoing protection of the town's water quality and preservation of a shellfish industry vital to the community's economic survival. In addition, the community is committed to promoting an extensive water conservation program. Activities in progress or already completed include conducting a leak detection survey of all residences, retrofitting commercial toilets, and implementing "xeriscape" landscaping.

WATER QUALITY MONITORING

The historic Suwannee River, immortalized by songwriter Stephen Foster, begins in Georgia's Okefenokee Swamp, and empties into the Gulf of Mexico near Cedar Key, one of the few remaining areas for shellfish harvesting in Florida. Designated an Outstanding Florida Water, the Suwannee River has managed to remain relatively free of the pollutants that have diminished the health and tarnished the beauty of many of our nation's waterways. Yet, even the Suwannee is in danger of becoming a casualty. In a stretch of river known as the Middle Suwannee, nitrate levels are at the highest level in 10 years. Animal waste and fertilizers from this rural region's many dairy and poultry operations are thought to be contributing factors, along with human waste from inadequate or poorly functioning septic systems, and fertilizers from other commercial and residential activities. To stem the tide of nutrient loading and other pollutants into this Outstanding Florida Water, state and federal agencies have joined in cooperative monitoring efforts to track the quality and quantity of water flowing through the river, its springs, and groundwater.

Recognizing that whatever flows into the Suwannee will eventually wash into the Gulf, efforts also are being made to monitor closely the condition of the state's coastal waters, vital to the survival of the state's fishing, shellfish, and tourism industries. Project COAST is one such monitoring project. Launched in 1997 by Florida's Suwannee River and Southwest Florida water management districts as a one-year water quality monitoring study, Project COAST is now an ongoing program coordinated by the University of Florida's Department of Fisheries and Aquatic Sciences in cooperation with the Florida Department of Environmental Protection and citizen volunteers. Using their own boats, trained volunteers take water samples at fixed sites adjacent to five coastal communities along more than 100 miles of the west central Florida coastline. They measure temperature, salinity, water clarity, chlorophyll concentrations, nitrogen, and phosphorous, and submit the data to the university for analysis. Results from this cost-effective sampling program will be used to develop a long-term data set, which will in turn be used to establish baseline water quality conditions for coastal waters. The results also will provide educational information concerning environmental issues to the public.

CREATING QUALITY COMMUNITIES

The tiny coastal village of Suwannee suffered a severe economic blow in 1991 when high bacterial contamination caused by the town's poor septic systems prompted the federal government to

close Suwannee Sound to oyster harvesting, the community's main industry. To help preserve and protect the area's water resources and revive the $1 million annual local shellfish industry, the Suwannee River Water Management District allocated $25,000 for a detailed feasibility study that addressed the town's wastewater treatment needs. The District also helped city and county officials obtain $9.7 million in federal grants and loans to finance the town's new wastewater treatment system, which is now up and running. The resounding success of that effort was the inspiration for what is now the Quality Communities Program.

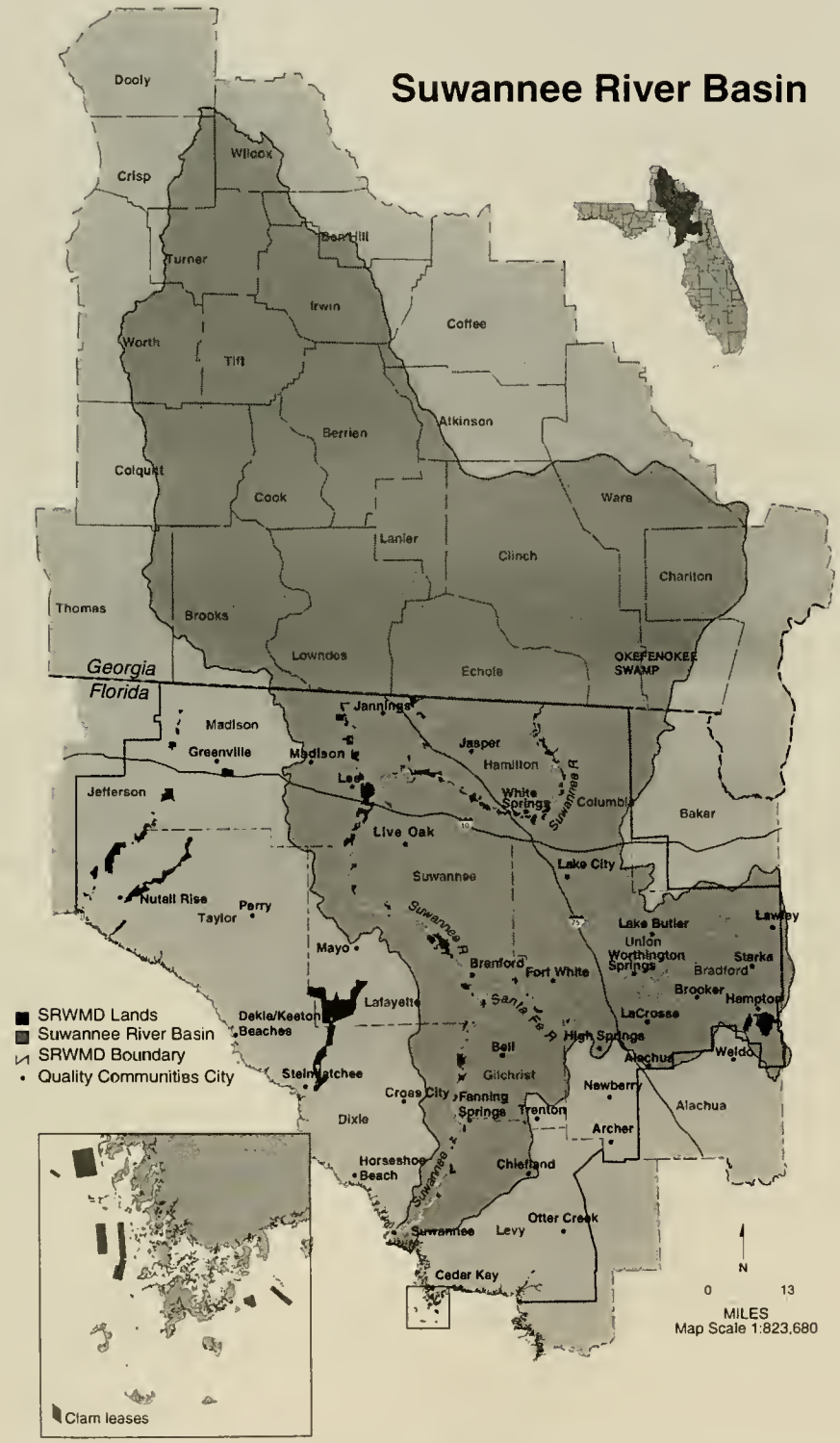

Figure 2. *Clamelot*, in relationship to the Suwannee River Basin and Big Bend coastline of north central Florida. Locations of shellfish aquaculture leases off Cedar Key, the boundaries of the Suwannee River Water Management District, land acquisitions of the Suwannee River Water Management District, and cities targeted for the Suwannee River Water Management District's Quality Communities Program are shown.

The goal of the Quality Communities Program is to help small rural communities protect water resources and at the same time improve their quality of life by offering technical expertise and funding needed to complete critical water quality and community infrastructure projects. Some of the state's poorest counties, in terms of per-capita income, education, property values, and taxable property levels, are located within the Suwannee River Water Management District. These counties lack the resources to make the necessary improvements to their drinking water supplies, stormwater drainage systems, and wastewater treatment facilities.

The District has targeted 37 communities and some unincorporated areas for assistance so that by the year 2010 each one will have the opportunity to become a Quality Community. The District will set aside $500,000 per year for 10 years as "seed money" for projects that will eliminate street and residential flooding, for preengineering or feasibility studies for project cost estimates, and as leverage for other available grants and funds. Additional funds from the District's land acquisition and management program will be used to purchase lands for stormwater storage and water supply protection.

LAND ACQUISITION AND MANAGEMENT

Florida's land and water resources are forever linked. To protect the rivers, lakes, streams, and underground water supplies, the lands around them must be managed properly. The Suwannee River Water Management District currently owns and manages nearly 100,000 acres of riverfront and wetlands to provide natural storage areas for flood waters, reduce loss of life and property due to floods, protect ground and surface water resources of the region, and protect natural systems associated with floodplain ecosystems. One of the District's key acquisitions in terms of coastal protection was the purchase of Atsena Otie, a 60-acre barrier island located near Cedar Key.

An island of great historical significance, Atsena Otie was the original site of Cedar Key and in the 1800s served as Army headquarters for General Zachary Taylor. It later grew into a prosperous city with a school, hospital, post office, and several sawmills, one of which belonged to the Eberhard Faber cedar pencil manufacturer. Around the turn of the century, residents gradually abandoned the island following a series of devastating hurricanes, and the island has since remained uninhabited. Today the island and its surrounding waters are home to a variety of animals, including egrets, ospreys, ibises, turtles, squirrels, raccoons, Gulf sturgeon, dolphins, and manatees. Cordgrasses fringe the island, and the interior is alive with sand live oaks, red cedars, cabbage palms, palmettos, and other vegetation.

The waters surrounding Atsena Otie may be harvested for shellfish, and clam lease sites are located on each side of the island. In the early 1990s, private developers introduced plans to build a residential community on the island. Concern over the potential impacts of septic tanks and stormwater runoff on the coastal environment and the local shellfish industry prompted the District to purchase the land in 1997 for $3.1 million, thereby placing it under public ownership.

Today the U.S. Fish and Wildlife Service manages the island. It is open to the public for swimming, hiking, fishing, and nature observation but not for camping. Posted signs remind visitors to carry out all of their trash, and a self-composting portable toilet has been placed on the island to accommodate visitors' needs and to reduce potential environmental impacts of human waste on the nearby clam lease sites.

PRESERVING AGRICULTURE, PROTECTING THE ENVIRONMENT

Residents of rural North Florida's Suwannee River Basin are struggling to balance and preserve the two things most vital to their economy and quality of life: clean and scenic natural resources and agriculture. This watershed features the Suwannee River and one of the largest concentrations of freshwater springs in the world. It also contains a large percentage of the state's farms, dairies, cattle, and poultry operations. When high nitrate levels were discovered in the Suwannee River, its springs, and local groundwater, the regional water managers sought the involvement of farmers, local governments, environmental regulators, and all citizen stakeholders in a collaborative effort to reduce nutrient loadings to the watershed, the waters of which eventually empty into the Gulf.

The Suwannee River Basin Nutrient Management Working Group, comprised of two dozen government agencies and independent organizations, was formed to facilitate that effort. Three technical committees, focusing on management of fertilizers, animal waste, and human waste, are gathering and coordinating information that will assist in the eventual design and implementation of a basinwide nutrient management plan. A program coordinator conducts public meetings and workshops, and serves as a liaison between agencies, agricultural interests, elected officials, and the public. Approximately $6.3 million in state and federal funds has been earmarked for voluntary, incentive-based, nonregulatory cost-share programs to initiate best management practices at farms; 43 dairy farmers and 102 poultry producers in the two most highly impacted counties will be able to participate.

INTERSTATE COORDINATION

Florida and Georgia share the 10,000 square-mile Suwannee River Basin, and the two states are working together to protect it. In 1996 the Suwannee River Water Management District, Florida Department of Environmental Protection, Georgia Department of Natural Resources (DNR), and U.S. Fish and Wildlife Service joined in an informal alliance to foster communication and cooperation between the two states and to develop a comprehensive plan for safeguarding the water resources within the basin. Without the use of interstate compacts or agreements, the agencies have made significant strides in the areas of cooperative monitoring, information exchange, and outreach. Alliance activities include public meetings and workshops, a semi-annual newsletter, and a satellite-image poster of the entire Suwannee River Basin.

Perhaps the most important accomplishment yet is the coordination of monitoring activities throughout the entire Suwannee River Basin. The Suwannee River Water Management District and the Georgia DNR Environmental Protection Division are performing monitoring on a parallel schedule, using the same parameters and methods at 72 sites in Florida and 73 in Georgia. Following completion of the testing for ammonia, nitrates, total phosphorous, fecal coliform, and trace metals, the agencies will publish a joint report under the auspices of the Alliance.

*"Don't let it be forgot that once there was a spot for one brief shining moment that was known as Camelot."**

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INCREASED DENSITY OF LARGE *RANGIA* CLAMS IN LAKE PONTCHARTRAIN AFTER THE CESSATION OF SHELL DREDGING

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ABSTRACT *Rangia cuneata* is a relatively large clam found in the oligohaline areas of Atlantic and Gulf of Mexico estuaries. *Rangia* is common in Lake Pontchartrain, Louisiana, and accumulated shells supported a mining industry from 1933 to 1990. Shells were used primarily for construction of roadways, parking lots, and levees, and in the production of cement. Based on mean densities, a 1954 study found that large clams (> 20 mm) were abundant (95/m^2); however, large clams were found to be less abundant in 1973 (39/m^2), 1982 (2/m^2), and 1984 (41/m^2) studies. Because baseline and comprehensive time sequence studies were not done, it is unclear whether shell dredging caused the differences in abundance. Fifty-five sites were sampled in 1996 and 1997 from lakes Maurepas, Pontchartrain, and Borgne to determine the abundance and distribution of *Rangia*. Lake Maurepas and Lake Borgne were included to provide information about *Rangia* in adjacent estuaries that have salinities lower and higher than Lake Pontchartrain. Data from the Lake Pontchartrain sites were used to determine whether the number of large clams had increased after shell dredging was stopped in 1990. Large clams were abundant at most sites (Lake Pontchartrain mean density 126/m^2), but absent in a 350-km^2 area affected by saltwater intrusion and hypoxic conditions from the Inner Harbor Navigation Canal (IHNC). Although large clams were absent from the eastern part of the lake in earlier studies, the highest density (602/m^2) was found in this area. Based on the current distribution and density of large clams, shell dredging had a significant impact on density and recovery has occurred since cessation of dredging.

KEY WORDS: *Rangia cuneata*, shell dredging, size classes, Lake Pontchartrain, density, distribution

INTRODUCTION

Lake Pontchartrain is an embayment in a large estuarine system in southeastern Louisiana. It has a mean salinity of 4 ppt, a mean depth of 3.7 m, and a surface area of 1,630 km^2 (Fig. 1). Saline water enters from adjacent estuaries through natural tidal passes and a navigation canal. Flow through the Inner Harbor Navigation Canal (IHNC) causes salinity stratification and episodic bottom hypoxia (Poirrier 1978, Junot et al. 1983, Schurtz and St. Pe 1984). Fresh water sources are streams and New Orleans area outfall canals. Fresh water also enters from the Mississippi River through the Bonnet Carre Spillway as leakage when the river is high, and when the spillway is opened to prevent possible flooding of New Orleans and other downstream communities (Barbe and Poirrier 1991). During this study, the spillway was open from March 17 to April 18, 1997.

Lake Pontchartrain is located north of New Orleans, and more than 1.5 million people live in its basin. With increasing urbanization in the New Orleans area over the last century, concerns about possible declines in water quality, fisheries, and recreational use of the estuary have been raised (Houck et al. 1989). A major environmental concern was adverse impacts from dredging for the shells of the common rangia, *Rangia cuneata* (Gray), from deposits in the estuary.

Shell dredging began in 1933 and was stopped in 1990. The volume of shells harvested increased until the mid 1970s and then declined (USACOE 1987). Shells were used for foundations, roadways, as an ingredient in many industrial products, and for oyster cultch. The shells harvested, according to estimates from the 1980s, had an annual gross value of $34 million. Dredging was allowed in 44% of the lake; it was prohibited near shorelines, bridges, and oil and gas wells and pipelines. Dredging was regulated so that only 1% of the bottom was disturbed at a time. Suction dredges drew up bottom sediment, and shells were separated from the sediment by washing on screens. The discharge of the sediment slurry directly into the lake increased turbidity near the dredge. Turbidity increases also were probably caused indirectly by shell dredging, which results in the formation of unconsolidated bottom sediments that are easily resuspended by wind. Besides increased turbidity, introduction of toxic substances from the sediment into the water column and disruption of the bottom invertebrate community were additional environmental concerns (USACOE 1987).

Early studies (GSRI 1974) on the effects of shell dredging did not show any significant environmental effects; however, studies by Sikora and Sikora (1982) found an average density of only 2/m^2 of *Rangia* > 20 mm. They attributed the low densities of large clams to the adverse effects of shell dredging. R. Darnell obtained information on the distribution and abundance of large *Rangia* (> 20 mm) during studies of Lake Pontchartrain in 1953–1954 (Suttkus et al. 1954). These data were unpublished but cited by Sikora and Sikora (1982) and later presented in an environmental impact statement prepared by the U.S. Army Corps of Engineers (USACOE 1987). Darnell found large *Rangia* clams were present at an average density of 95/m^2 (USACOE 1987). Other studies that included information on the abundance and distribution of *Rangia* in Lake Pontchartrain were Tarver and Dugas (1973) and Poirrier et al. (1984), who found large clam average densities of 39/m^2 and 41/m^2, respectively. These studies also found that few clams occurred in the areas of the lake that were dredged. It was not clear whether the high numbers of large clams reported by Darnell (USACOE 1987) were indicative of the natural condition of Lake Pontchartrain in the 1950s, because shell dredging began in 1933 and management practices were thought to be sufficient to avoid a severe impact on Lake Pontchartrain.

Rangia cuneata is generally found in estuaries from the upper Chesapeake to Vera Cruz, Mexico (LaSalle and de la Cruz 1985). It can make up 95% of the benthic biomass in low salinity estuaries (Cain 1975). It is a nonselective filter feeder that is important in converting energy from suspended organic material to clam biomass (Gaston et al. 1996). Juvenile and adult *Rangia* provide food for fish, duck, and invertebrate predators (Darnell 1961, LaSalle and de la Cruz 1985). *Rangia* clams, which are dioecious, shed gametes directly into the water. A rapid change in salinity of 5 ppt

can trigger spawning (Cain 1975). In Lake Pontchartrain, clams become sexually mature at about 24 mm (about 2-years-old), and spawning peaks occur from March to May and from late summer to November (Fairbanks 1963). Adult *Rangia* can withstand a salinity range of 0 to 25 ppt (Hopkins et al. 1973). However, permanent populations do not occur at salinities above 15 ppt (Hopkins et al. 1973). Filtration rates are highest at 5 ppt and decrease with increasing salinity (Holley and Foltz 1987).

This study was conducted to determine whether there has been an increase in the density of large *Rangia* after shell dredging was banned in the summer of 1990. A more detailed description of this study is presented in Abadie (1998). If the density of large clams had increased significantly, it would indicate that shell dredging had a significant effect, and that recovery from the adverse impact had occurred. Francis and Poirrier (1998) showed an increase in Secchi disc transparency after shell dredging was stopped. This water quality improvement may be related to an increase in the density of large clams.

MATERIALS AND METHODS

Lakes Maurepas, Pontchartrain, and Borgne were sampled to determine the abundance and distribution of *Rangia* (Fig.1). Lake Maurepas, which has a mean salinity of 0.5 ppt, and Lake Borgne, which has a mean salinity of 12 ppt (Perret et al. 1971), were included to provide information about *Rangia* in adjacent estuaries with salinities lower and higher than Lake Pontchartrain. Six sites in Lake Maurepas, five sites in Lake Borgne, and 44 sites in Lake Pontchartrain were sampled from November 1995 to October 1997 (Fig.1). These sites were sampled once to obtain information on the spatial distribution of large *Rangia*. Temporal (seasonal) information was obtained by quarterly sampling of five sites on an east-west transect that extended through the middle of the Lake Pontchartrain (Fig.1). Additional samples from this transect were taken to study the effects of the March 17, 1997, Bonnet Carre Spillway opening. As a result, it was sampled seven times: November 11, 1996, March 12, 1997, April 30, 1997, June 6, 1997, July 28, 1997, September 17, 1997 and October 29, 1997.

Three replicate samples were collected from each site with a (15 × 15 cm) petite Ponar dredge. Each sample was emptied into a 0.6-mm sieve bucket to separate organisms from fine materials in the sediment. All material retained on the sieve was transferred from the bucket into 500-mL specimen jars and fixed in a 10% buffered formalin solution with rose bengal. In the laboratory, the sample was washed on a 0.5-mm sieve to remove formalin and any remaining sediment that could pass through the sieve. Each sample was examined with a Wild M-5 dissecting microscope to remove *Rangia* from remaining sediment. The *Rangia* specimens were measured to the nearest millimeter and the lengths were grouped

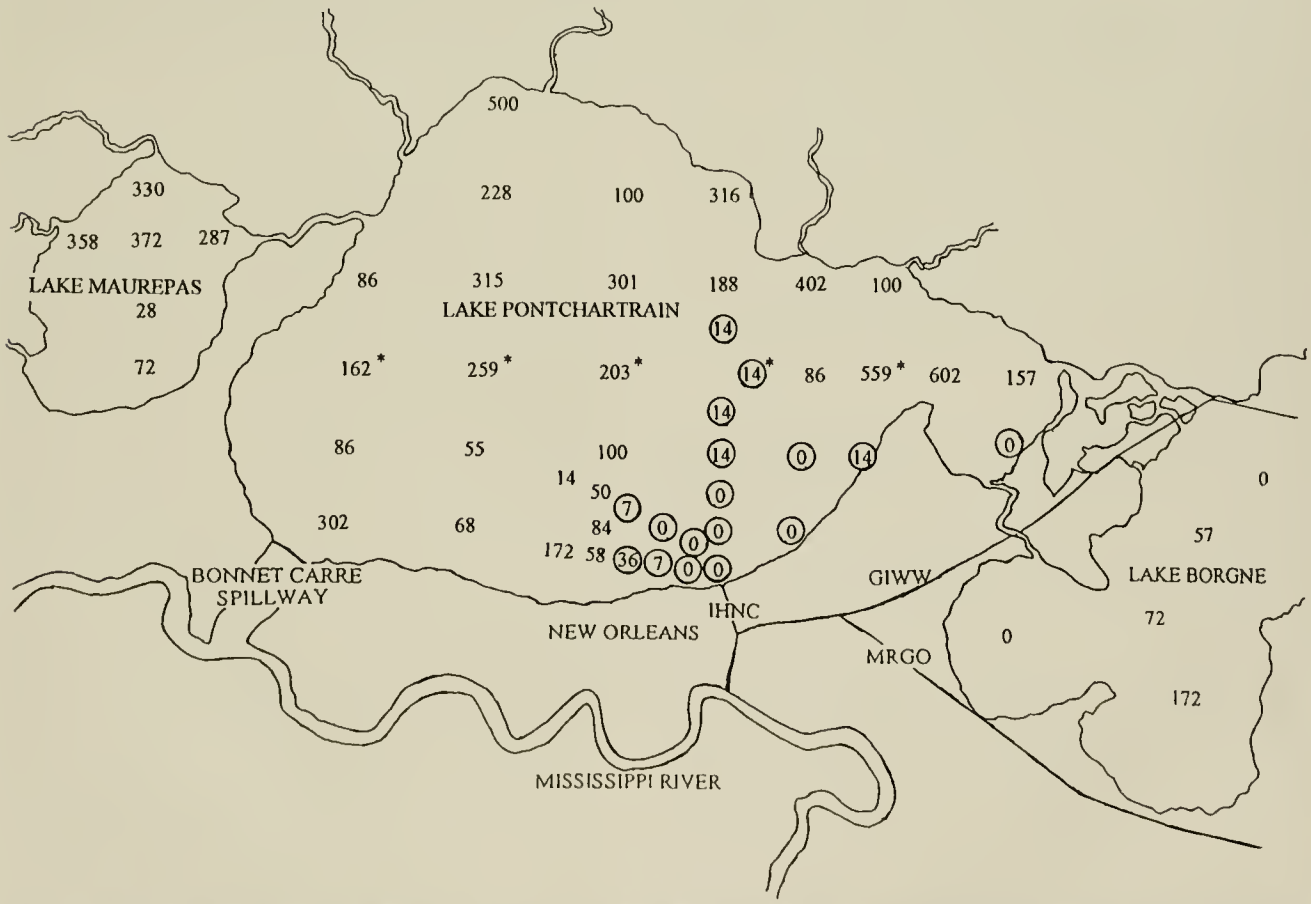

Figure 1. Map of lakes Maurepas, Pontchartrain, and Borgne, Louisiana. Mean density of large (> 21 mm) *Rangia cuneata* are indicated at each of the 55 sites sampled from November 1995 to October 1997. The five sites along the east-west transect, sampled seasonally, are marked with an asterisk. Mean densities that are circled signify sites that are significantly different from the four sites on the east-west transect not affected by hypoxia.

into 5-mm increment size classes (0–5, 6–10, 11–15, 16–20, 21–25, 26–30, 31–35, 36–40, and 41–45 mm).

Surface and bottom water temperatures, salinity, and dissolved oxygen were measured concurrent with clam sampling using a YSI model 85 meter. A weighted 20-cm Secchi disc was used to measure transparency and depth.

All historic data (Tarver and Dugas 1973, Sikora and Sikora 1982, Poirrier et al. 1984, USACOE 1987) were converted to N/m^2. The designation of large clams, equaling 20 mm or more in length, was made in the baseline study by Suttkus et al. (1954), and was used to make comparisons among studies. A one-way ANOVA was used to test the following null hypotheses: (1) there has been no increase in large clam density since shell dredging was stopped; (2) there has been no change in the density of clams in the 0–10 mm and 11–20 mm size classes in the studies by Sikora and Sikora (1982), Poirrier et al. (1984), and this study; and (3) there were no temporal or spatial differences in the distribution of large clams.

RESULTS

Water depth at the study sites ranged from 1.8 m at the nearshore stations to 6 m near the Inner Harbor Navigation Canal, and averaged 4 m. Surface salinity ranged from 0.1 ppt in Lake Maurepas to 8.9 ppt in Lake Borgne. The salinity of Lake Ponchartrain decreased to freshwater conditions after the March 1997 Bonnet Carre Spillway opening, and returned gradually to pre-opening conditions by October 1997. Secchi disc transparency ranged from 8 cm after the Bonnet Carre Spillway was opened to 290 cm in the fall near the north shore. Water quality measurements followed known seasonal trends (Francis et al. 1994) and known responses from past Bonnet Carre Spillway openings (Poirrier and Mulino 1977). Specific effects of the 1997 spillway opening on *Rangia* and other benthic invertebrates will be included in reports from other ongoing studies.

The number of *Rangia* greater than 21 mm/m^2 from each site is presented in Figure 1. The density of large clams in Lake Ponchartrain ranged from zero in the southeastern region, near the IHNC, which is subjected to salinity stratification and episodic hypoxia, to 602/m^2 in the eastern lobe, and averaged 126/m^2 for all areas. The average density of large *Rangia* from the three lakes was 137/m^2. A comparison of average densities (N/m^2) found by this study with densities found by previous studies (USACOE 1987, Tarver and Dugas 1973, Sikora and Sikora 1982, Poirrier et al. 1984) indicates high densities in the 1954 study, low densities in the 1973, 1982, and 1984 studies, and recovery in this study (Abadie 1998) (Fig. 2). Results of the one-way ANOVA showed that there was a significant difference in the number of large clams among the studies ($P < .0001$). The average densities for the 1954 study and the 1998 study were significantly different from the 1973, 1982, and 1984 studies, but there were no significant differences between the 1954 and 1998 studies (Fig. 2).

An analysis of variance showed that there were statistically significant differences in the density of clams >21 mm among the Lake Pontchartrain sites. A means comparison for each of the 55 sites was performed and analyzed against four of the five sites on the east-west transect. Sites on the east-west transect were used in this comparison, because data from them was based on a total of 21 replicate samples, contained seasonal information, and ranged over a salinity gradient. One site on the east-west transect was excluded from the comparison; this site was north of the IHNC and was known to experience by episodic hypoxia (Schurtz and St. Pe 1984). Sixteen sites, located near the Inner Harbor Navigation Canal, were significantly different from the remaining four east-west transect sites (Fig 1).

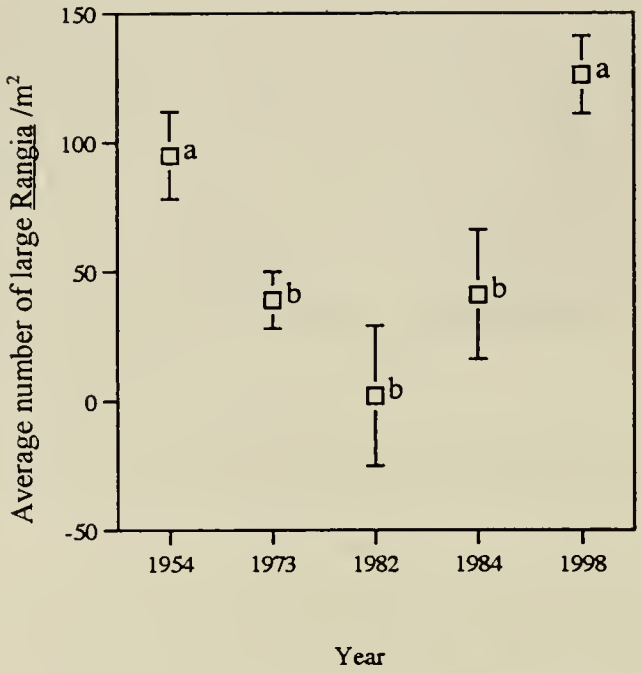

Figure 2. Historic average densities and standard error of large *Rangia cuneata* in Lake Pontchartrain. Historic averages were complied from USACOE (1987), Tarver and Dugas (1973), Sikora and Sikora (1982), Poirrier et al. (1984), and this study. A one-way ANOVA and means comparison were used to assess the effect of year on the average density of large *Rangia* in Lake Pontchartrain. Means with different letters are significantly different at the .05 level.

A one-way ANOVA was used to compare the densities of 0–10 and 11–20 mm size classes (Abadie 1998) with two earlier studies (Sikora and Sikora 1982, Poirrier et al. 1984). There were significant differences in the average densities of 0–10 mm clams among the 1982 (3,164/m^2), 1984 (266/m^2), and 1998 (278/m^2) studies, but no differences were found between the 1984 and 1998 studies ($P < .0001$). No significant differences ($P = 0.7620$) were found in the densities of 11–20 mm size class among the three studies: 1982 (31/m^2), 1984 (22/m^2), and 1998 (57/m^2).

The distribution of size class frequencies for all clams collected from lakes Maurepas, Pontchartrain, and Borgne was bimodal (Fig. 3). The clams ranged from 0 to 45 mm and were divided into 5-mm size classes. Peaks occurred in the 0–5 mm and the 26–30 mm size classes. The 0–5 mm size class made up 41% and the 26–30 mm size class 21% of the total clams collected. The percentages in the other sizes classes were 6–10 mm size class 16%, 11–15 mm size class 4%, 16–20 mm size class 1%, 21–25 mm size class 10%, 31–35 mm size class 6%, and the 36–40 and 41–45 mm size classes 1% each.

The analysis of the seven, seasonal east-west transect samples (Fig. 1) showed significant spatial and temporal differences were in the 0–5, 6–10, and 11–15 mm size classes; significant spatial differences were present only in the 21–25, 26–30, 31–35 and 36–40 mm size classes among the five sites. There were no clear trends related to seasons or differences among sites for clams < 21 mm. The introduction of Mississippi River water through the Bonnet Carre Spillway may have affected trends. The density of large clams (> 21 mm) was lowest at site four, where *Rangia* may have been affected by hypoxia, and greatest at site five.

Figure 3. *Rangia cuneata* size class frequencies from 55 sample sites in lakes Maurepas, Pontchartrain, and Borgne, Louisiana.

DISCUSSION

A comparison of the density of large *Rangia* from prior studies (Tarver and Dugas 1973, Sikora and Sikora 1982, Poirrier et al. 1984, Darnell in USACOE 1987) with current data (Abadie 1998) shows there was a significant decrease from the early 1950s through the 1980s in the density of large *Rangia*. The comparison also revealed that current densities are not significantly different from those found in Darnell's 1954 study (USACOE 1987) (Fig. 2). The decrease in the density of large *Rangia* appears to be related to the intensity of shell dredging, and the best explanation for the increase in the density of large clams is the cessation of shell dredging since the summer of 1990. When shell dredging began in 1933, about 0.25 million cubic yards of shells were harvested per year. Until 1956, production was less than 2 million cubic yards per year. Production increased after 1956, peaked at 7.5 million cubic yards in 1976, and then declined (USACOE 1987). Many live *Rangia* clams were harvested in the early years of production, but by the early 1970s, the number of live clams decreased (Tarver 1972). Although restrictions were in place, a comparison of current data with historic data confirms that shell dredging had a significant effect on the density of large clams.

The severity of the impact of shell dredging on Lake Pontchartrain was unclear (GSRI 1974, USACOE 1987) because no studies were conducted before 1933, when dredging started. The only early quantitative data on benthic invertebrates was the density of large clams from Darnell's studies during 1953 and 1954 (USACOE 1987). These limited data may not have been adequate to characterize past conditions, and the contribution of other perturbations such as urban runoff and saltwater intrusion to any decrease in clam density was unknown. Direct removal of large clams by dredging was not thought to produce an effect because, with the restrictions in place, it was supposed to take up to 4 years to disturb an area equivalent to the area permitted for dredging. However, some areas were disturbed more frequently than others (USACOE 1987). Sikora and Sikora (1982) suggested that dredging decreased sediment bulk density and large clams sank into the less dense sediment. However, experiments by Taylor Biological Co. indicated that clams could maintain their position in Lake Pontchartrain's sediment bulk densities (USACOE 1987).

The absence or low densities of large clams from sites north of the Inner Harbor Navigation Canal (IHNC) indicates that episodic hypoxia from salinity stratification due to salt-water intrusion (Poirrier 1978, Junot et al. 1983, Schurtz and St. Pe 1984) affects the establishment of older, larger *Rangia*. Based on the sites that had a statistically significant difference in the density of large clams, a 350-km^2 area is affected (Fig 1). The IHNC was completed in 1963, and changes in the salinity regime and episodic hypoxia occurred along with the effects of shell dredging. The combined effects of shell dredging and salt-water intrusion may have contributed to the lakewide reduction of large clams. Shell dredging produced trenches that were about 2 m wide and 1 m deep. These trenches were generally backfilled by sediment discharge. However, when they were not completely filled, they may have distributed saline, hypoxic waters from the area near the IHNC to other areas of the lake.

Many large *Rangia* were found in the eastern lobe of Lake Pontchartrain, and relatively high densities were found in the more saline waters of Lake Borgne (Fig. 1). No large *Rangia* were reported from the eastern lobe of Lake Pontchartrain in past studies, including Darnell's 1954 study (USACOE 1987). This is the first quantitative report of *Rangia* densities from Lake Borgne.

Sikora and Sikora (1982) reported average lakewide densities of 3,164/m^2 for 0–10mm *Rangia*, which were much higher than the average of 266/m^2 reported by Poirrier et al. (1984), or the average of 278/m^2 found in this study (Abadie 1998). Sikora and Sikora's (1982) mean densities were higher than the highest site densities of 902/m^2 and 1,568/m^2 reported by Poirrier et al. (1984) and this study (Abadie 1998). More small clams may have been present in the past due to lack of competition from large clams, the numbers of which were reduced from dredging activities. However, this is not supported by the comparable values reported by Poirrier et al. (1984).

The current *Rangia* population shows a bimodal distribution (Fig. 3), indicating heavy recruitment may occur continually (Fairbanks 1963). Intermediate size classes are found in lower frequencies due to intense predation pressure (Darnell 1961). As clams increase in size, growth rate slows, predation pressure drops, and a stable peak of larger clams is evident.

In the analysis of the large size classes over time along the east-west transect, two trends are clear. The number of large clams at each site remains stable throughout the year. Thus, the 1997 spillway opening probably did not have a deleterious effect on the density of large clams. The second apparent trend is the low number of large clams through the year at the site north of the IHNC. Low densities of large *Rangia* were also found at 15 other sites in the vicinity due to episodic hypoxia (Fig. 1).

The increase in Secchi disc transparency after shell dredging was stopped (Francis and Poirrier 1998) may be related to the increase in density of large clams. It is apparent that large clams have returned to north shore and mid lake sites. However, densities remain at low at south shore sites due to the effects of the IHNC. Francis and Poirrier (1998) found water clarity at both a north shore and a mid lake site was better than at a south shore site. Therefore, the high filtration rate associated with high densities of large *Rangia* may help improve general water quality.

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EVALUATION OF A NEW TAGGING TECHNIQUE FOR MONITORING RESTORATION SUCCESS

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ABSTRACT Venerid clams, *Austrovenus stutchburyi*, were tagged with small aluminum discs, enabling relocation using a metal detector. Tag loss varied between treatment types, being highest for small, densely packed clams. Over three sites the mean tag loss across all treatments was 10% (± 2.87) after 7 months. This is likely to be an overestimate as only a subsample of individuals was recaptured. Laboratory studies showed no significant difference in survival, growth, or condition between tagged and untagged clams. Ability to rebury was not affected by tags; all tagged and untagged individuals burrowed within 24 h of being placed in tanks. The technique was also found effective for a deeper burrowing tellinid bivalve. The extensive movement of four whelk species made relocation difficult, but the technique still holds potential for the tag and recapture of these gastropods. Studies previously considered difficult are feasible with this technique.

KEY WORDS: *Austrovenus stutchburyi*, clam, restoration, metal detector, monitoring, tag and recapture, New Zealand.

INTRODUCTION

Austrovenus stutchburyi (Wood 1828) is a shallow-burrowing, filter-feeding clam found in sheltered, soft-shore, intertidal habitats around New Zealand. Adult clams have an average shell length of 30–40 mm. Populations of *A. stutchburyi* are vulnerable to increased sedimentation from coastal development and overharvesting. Consequently, this popular resource has declined at many locations throughout New Zealand, although the extent of this decline has only recently been recognized. Our research investigates the potential for restoration of infaunal clams through studies of the ecology of *A. stutchburyi*; the study included manipulative field experiments to assess movement patterns, predation rates, and responses to translocations. Despite being used increasingly overseas, restoration is a novel technique for New Zealand.

Restoration requires monitoring of biological parameters to determine success (Pratt 1994). In the long term, reproductive output and the ability to establish self-maintaining or sustaining populations are the most critical considerations, but in the initial stages of shellfish enhancement the important parameters are survival and growth. Estimates of these can be obtained at the population level by using cohort analysis or through the analysis of growth rings (Lutz and Rhodes 1980). More direct estimates can be obtained by tag and recapture techniques (Brousseau 1978, 1979; Craig 1994). In high latitude marine environments, bivalves often lay down annual rings that correspond to seasonal growth spurts (usually in summer). This phenomenon has been reported for some *A. stutchburyi* populations in southern New Zealand (Coutts 1974, Marsden and Pilkington 1995), but many other studies have shown that shell rings are too variable to be relied upon for calculations of age or growth (Larcombe 1971, Coutts 1974, Blackwell 1984, Martin 1984). Tag and recapture procedures are considered more likely to provide accurate estimates of survival and growth for this species.

There are several methods of externally marking shells for later recapture of known individuals; for example, paint (Dobinson et al. 1989), alizarin red, a calcium stain (Peterson et al. 1995), or numbered tags. New Zealand bivalves commonly been tagged with numbered plastic tags glued to the shell (e.g., *A. stutchburyi*, Martin 1984, *Paphies subtriangulata*, Grant 1994, *P. australis*, Hooker 1995). The tag and recapture technique requires a reasonable recapture rate, which can be difficult to achieve for infaunal bivalves. Conventionally tagged animals often migrate out of the area where they were released and it takes considerable time and effort to sieve through large amounts of sediment to find them. This also makes estimates of mortality difficult, as there is no way of knowing how many tagged animals were missed.

One way to avoid this is to cage bivalves on the shore, which is a common experimental technique (Hurlberg and Oliver 1980; Virnstein 1980; Martin 1984). However, this procedure is difficult to implement on beaches visited by large numbers of people and in areas impacted by harvesting. In soft-sediment habitats, cages can influence water flow and sedimentation in experimental plots and these effects must be assessed using appropriate controls (Hurlberg and Oliver 1980). A new technique with the potential to avoid these problems tags bivalves with small aluminum tags glued to the shell, and relocates them using a highly sensitive metal detector run over the surface of the sediment. This technique was pioneered in South Africa for the highly mobile surf clam, *Donax serra* (Dugan and McLachlan, 1999). Tag loss in their study was around 4% and tagging was found to have no significant effect on condition or behavior. Such a tagging method would enable *A. stutchburyi* to be relocated over a wide area, without caging. Prior to this, Neves et al. (1989) tested techniques of telemetry on the freshwater mussel *Actinonaias ligamentia*. Using epoxy resin, magnets were secured to the valve, the mussels were placed at known locations, and a systematic search conducted with a magnometer. The degree of successful relocation of tagged mussels in their study was not given.

Estimation of growth and mortality depends on the assumptions that tagging does not affect behavior, increase the probability of predation or disease, or negatively effect growth or longevity (Southwood 1966). Many infaunal bivalves, including venerids, are well suited to external tagging because they are hardy, have a heavy shell (a tag therefore adds little weight), are reasonably large (tags can therefore be positioned so as not to interfere with opening or closing of the valves or protrusion of siphons), and their burrowing behavior means tags are not visible to predators.

This paper evaluates the potential use of aluminum tags and a

metal detector in tag and recapture studies of *A. stutchburyi*. Field and laboratory studies were used to test assumptions about tag loss (as recommended by Trebble et al. 1993) and the effects of tags on clam condition and behavior (as recommended by Martin 1984). Preliminary trials were also carried out on co-occurring bivalves and whelks.

METHODS AND MATERIALS

Tagging

Austrovenus stutchburyi were collected at low tide as this is when the clams are accessible in the field and least active (Beentjes and Williams 1986; Williams et al. 1993). After blotting with a paper towel, the clams were air-dried to provide a clean dry surface for attachment of tags. Aluminum tags were attached to the valve away from the apex and shell margin, using a clear, two-part epoxy resin (Araldite). Tags were 1 × 5 × 5 mm and weighed 69 mg (± 1.7 mg). All clams were also given a second tag consisting of a dot of enamel paint, which had been found previously to remain on the shell for at least 10 months. This allowed later estimates of the rate of loss of the aluminum tags. Enamel paint rather than plastic tags, was used because of the large number of shellfish in the experiment. After the glue had hardened and set, tagged clams were returned to the tank, prior to use in various experiments. The large clams were removed from the water for approximately 1.5 h in total and smaller clams for a shorter time.

Assessing Tag Effect

Tagged (treatment) and untagged (control) *A. stutchburyi* were kept in aquaria (320 × 250 × 150 mm) and monitored to determine the effect of tagging on mortality and growth. A 3-cm layer of sediment was placed in each aquarium prior to adding clams in order to mimic the natural environment. Sediment had been sieved through 2-mm mesh sieve to remove large macrofauna. Each aquarium held 10 small clams (10–18mm) and 10 large clams (25–32mm). Clams in control aquaria were subject to the same drying process as those tagged. Before being placed in the aquaria, all clams were measured (to the nearest 0.1 mm) and weighed before and after the addition of tags. Three randomly positioned replicate treatment and control aquaria were used.

The burrowing behavior of tagged and untagged clams was observed over the first 48 h. Aquaria were checked for mortality weekly, and clams were re-measured monthly. After 5 months, the physiological status of the tagged and untagged clams was compared using condition indices. To ensure that the potential effects on different components of the condition analyses were detected, three separate indices were used. These were dry weight condition index (CI-dry), gravimetric condition index (CI-grav), and body condition index (BCI).

$$\text{CI-dry} = \frac{\text{dry tissue weight (g)} \times 100}{\text{shell weight (g)}}$$

(Crosby and Gale 1990, Marsden and Pilking 1995)

$$\text{CI-grav} = \frac{\text{dry tissue weight (g)} \times 100}{\text{internal shell cavity capacity (g)}}$$

Where internal shell cavity capacity = total whole live weight − dry shell weight (Crosby and Gale 1990)

$$\text{BCI} = \frac{\text{dry tissue weight (g)} \times 100}{\text{shell cavity volume}}$$

Where volume = $\pi/6$ (shell height × length × breadth) (Savari et al. 1991)

Tag Loss and Tag Relocation

Loss of aluminum tags was assessed both in the laboratory experiment on tag effect and in the field as part of a transplant experiment. Field studies were carried out at two intertidal sites, Point Wells and Lews Bay in the Whangateau Harbour near Leigh, in northeastern New Zealand (Fig. 1). A total of 4500 cockles were double-tagged and returned to the Whangateau Harbour (see Fig. 1), where they were transplanted to three separate sites (two at Point Wells and one at Lews Bay). Each group of 1,500 clams consisted of equal numbers of small (10–18 mm) and large (27–35 mm) individuals. Within each size category, the clams were transplanted into either packed, high-density plots (200 clams/0.25 m^2) or spaced out, low-density plots (50 clams/0.25 m^2). There were three replicates of each combination of treatments (clam size and density), giving an orthogonal multifactorial design. Tag loss was assessed during the experiment from marked cluckers (empty valves still attached at the hinge) retrieved in visual searches at the transplant sites (conducted weekly where possible). The assumption was made that tag loss from cluckers was representative of tag loss from live cockles.

The transplant experiment enabled the effectiveness of tag relocation to be assessed. A metal detector (Minelab sovereign XS) was moved across the sediment surface and when a tag was detected (signalled by an increased tone), the area was marked and the sediment was carefully excavated to expose the tagged clam.

Applications to Other Species

Preliminary trials were conducted to test the effectiveness of the aluminum tagging methodology for *Macomona liliana* (Iredale 1915) and several species of whelks. *M. liliana* was chosen for comparison with *A. stutchburyi* as it is found in similar habitats, but it is deep-burrowing, living approximately 20 cm below the surface. Whelks were chosen to test the method on co-occurring species that are highly motile.

Macomona liliana were collected from the field and then tagged using the same method as for *A. stutchburyi*. They were

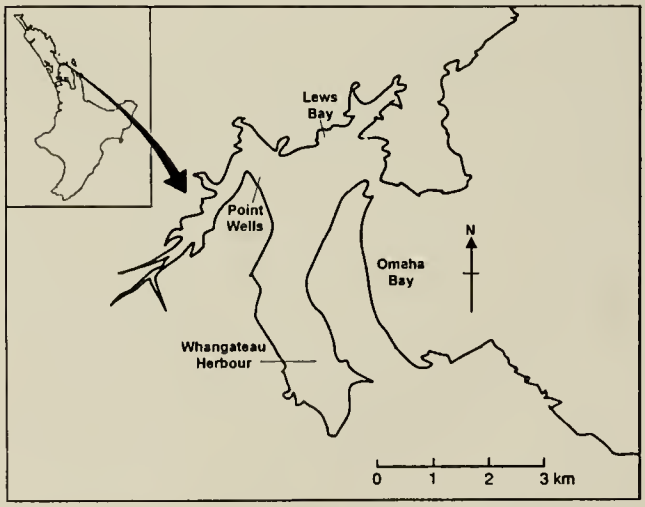

Figure 1. Transplant experiments using the aluminum tags were conducted at Lews Bay and Point Wells in the Whangateau Harbour (≅ 36°26′S, 174°46′E) in northeastern New Zealand.

held in salt-water, flow-through tanks overnight and then returned to the field on the next low tide. Three replicate plots were set up, each containing 20 adult *M. liliana*. These plots were checked regularly using the metal detector.

Four species of whelks were collected from the field. These were tagged using the same method as for *A. stutchburyi* and released in the same area they were collected from. The release site had a permanent marker from which movement by whelks could be calculated. This tag and release process was repeated twice. On the first, 15 each of *Cominella maculosa* (Martyn 1784), *C. adspersa* (Brugiere 1789), *C. virgata* (Adams 1863), and *C. glandiformis* (Reeve 1847) were tagged. On the second occasion, 30 *C. maculosa*, 50 *C. glandiformis*, 20 *C. virgata*, and 11 *Lepsiella scobina* (Quoy and Gaimard 1833) were tagged. As with the clams, the metal detector was swept over the sediment surface to locate the whelks. When a whelk was detected the location was marked with a plastic straw. When no more whelks were detected, each whelk marked by a straw was identified and recorded.

RESULTS

Tag Effect

Ability to rebury was not affected by the tags. All tagged and untagged *A. stutchburyi* burrowed within 24 h of being placed in laboratory tanks, and they remained burrowed for the entire experiment.

Mortality was not significantly different between tagged and untagged *A. stutchburyi*, for both the small and large clams (Fig. 2). However, there is an apparent difference in mortality between small, tagged clams and control clams. But the fact that the trend is toward higher mortality for control clams (36.7% versus 16.7% for tagged clams), certainly does not indicate an effect of the tag and is most likely due to problems with water supply. A two-way ANOVA (data pooled across tanks) showed no significant difference in mortality between treatments (P = .3336) or sizes (P = .0736) and no treatment * size interaction (P = .3336).

Although there was no obvious affect of the tagging procedure on mortality, there may have been a more subtle, sublethal impact. This was investigated by examining three indices of physiological condition. Three-way ANOVAs (treatment, size of cockle, tank) for each index revealed there were no significant interaction terms (P < .05 for all three indices). There was no significant tank affect

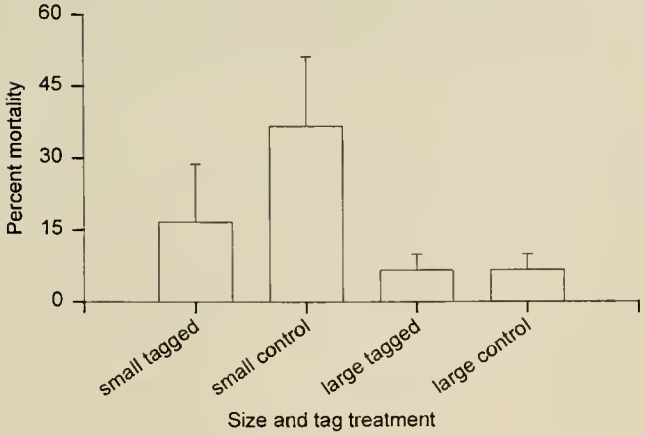

Figure 2. Percent mortality of tagged and untagged clams after 5 months. Error bars are standard error. (n = 3).

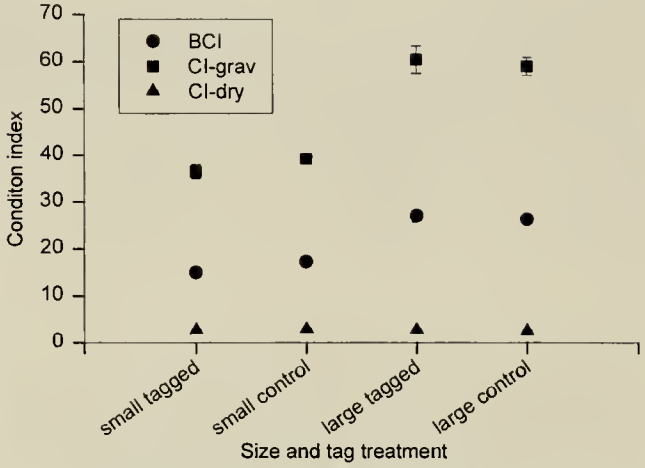

Figure 3. Condition indices for tagged and untagged clams after 5 months in the labnratnry. Error bars are standard errors. (n = 14 small clams. n = 26 large clams). Replicates have been pooled.

and therefore data were pooled across the three replicate tanks for graphical representation (Fig.3). There was no significant effect of treatment (P < .05 for all three indices), for tagged clams versus control clams, which was the comparison of interest. There was, as expected, a significant effect of size for all three indices, an artifact of the indices used.

There was no difference in growth between tagged and untagged clams, principally due to the fact that the clams did not grow significantly over the study period (Table 1). Dobbinson et al. (1989) also found a lack of growth for *A. stutchburyi* within the time frame of their experiment. Tagged clams that had been in the field for nearly a year were just beginning their summer growth spurt, with small clams having grown 2 mm or more over the months of September to October. This suggests that the tag had little, if any, effect on growth, even for small clams.

Tag Loss and Tag Relocation

No tag loss occurred during the 5 months that *A. stutchburyi* were held in the laboratory. Mean tag loss in the field over three sites, across all treatments, was 10% (± 2.87). Because of the low

TABLE 1.

Initial and final mean size of tagged and control clams, used in laboratory experiments investigating tag effect.

		Month 1		Month 5	
Size	Treatment	Mean	SE	Mean	SE
Small	Tag	15.4	0.65	15.2	0.65
Small	Tag	14.8	0.66	14.5	0.58
Small	Tag	15.3	0.54	15.3	0.65
Small	Control	15.3	0.73	15.1	0.11
Small	Control	15.3	0.65	15.8	0.70
Small	Control	15.0	0.67	15.5	0.76
Large	Tag	29.6	0.56	29.3	0.72
Large	Tag	29.3	0.59	29.1	0.64
Large	Tag	28.9	0.80	28.8	0.81
Large	Control	29.8	0.63	29.9	0.70
Large	Control	29.7	0.62	29.2	0.57
Large	Control	29.1	0.48	29.3	0.59

TABLE 2.

Relocation rate for tagged whelks in the field.

Day	Total Relocation All Species n = 111	*C. virgata* n = 20	*C. maculosa* n = 30	*C. glaudiformis* n = 50	*L. scobina* n = 11
2	10.8%	20%	3%	10%	18%
6	1.8%	30%	0%	2%	18%
10	0%	0%	0%	0%	0%

mortality of transplants (and therefore low numbers of cluckers retrieved), tag loss among treatments could not be compared statistically using this method. However, observational data suggest tag loss was greatest for small, densely packed clams. Although experimental plots were never permanently marked, it was possible to relocate plots on every sampling occasion. Relocation was accurate enough to avoid disturbing large areas of sediment.

Applications to Other Species

Macomona liliana were successfully relocated in the field after 2 months and there was no evidence of mortality for tagged individuals in the field. There has been a low return rate for the whelks, which is attributed to them moving away from the area, between tides, too quickly to be tracked. On the first sampling, only 16% of all whelks were relocated 1 day after release. All of these were either *C. adspersa* or *C. maculosa*. Three days later only one or two whelks were relocated. On the second sampling, there was a 4.5% incidence of tag loss before release. After 2 days, 10.8% of all whelks were relocated. Relocation rates were highest for *C. virgata* (20%) and *L. scobina* (18%) (Table 2). After 6 days, total relocation was only 1.8%, but relocation for *C. virgata* increased to 30% and remained at 18% for *L. scobina* (Table 2). After 10 days no whelks were detected. During this experiment, the whelks that were relocated were invariably solitary individuals, illustrating that the metal detector was sensitive enough to pinpoint a single tag approximately 5 cm under the sediment surface.

DISCUSSION

The aluminum tags had no detectable effect on growth, mortality, or behavior of *A. stutchburyi*. In addition, no effect on condition was found for three separate condition indices, including the gravimetric condition index (CI-grav), which is the recommended condition index to assess whether animals have been under stressful conditions (Crosby and Gale 1990). However, the time of year when tags are attached may affect growth and condition. Growth for shellfish is often seasonal, and attaching the tags during a period of high growth may have more effect than attaching them at another time of year. A long-term study of tag effect is required to investigate this, but the laboratory studies conducted here (in summer) suggest that any effect is likely to be small, irrespective of season. The advantage of using aluminum for the tag is that it is light enough not to affect behavior and is rust-resistant in saline conditions.

The fact that no clams held in the laboratory lost their tags may have been due to an absence of abrasive forces such as currents and the movement of abutting shellfish (which would be experienced in nature). These laboratory trials do confirm that the glue and tag are able to remain bonded to the shellfish in salt water for at least 5 months. Tag loss in the field was relatively low and did not reduce the effectiveness of the method. Because *A. stutchburyi* were in clusters, only a few tags were required to locate the plots. The metal detector is sensitive enough to locate a single tagged clam, but as no individuals moved away from the experimental plot this was unnecessary. In terms of evaluating the performance of restoration, this technique worked well for the ongoing monitoring of experimental transplants. With minimal effort it was possible to relocate experimental plots without the necessity of permanently marking them. For a full-scale restoration project, the time and effort required to tag all individuals would obviously be prohibitive. However, the technique would still work well if even a small proportion of the seed shellfish for enhancement were tagged for ongoing monitoring and treated as representative of the population. Also, this technique holds potential for ecological studies that seek to provide more information on which to base decisions about restoration alternatives.

The technique pioneered by Dugan and McLachlan (1999), and further developed in this paper, has allowed the recapture and tracking of bivalves. Dugan and McLachlan (1999) were able to

TABLE 3.

Burial depths of some common intertidal New Zealand soft-sediment bivalves.

Species	Common Name	Maximum Size	Burial Depth	Reference
Macomona liliana	wedge shell	50–60mm	≤20cm	Morton & Miller (1973)
Austrovenus stutchburyi	cockle	30–40mm	Top few cm	Morton & Miller (1973)
Nucula hartvigiana	nut shell	<10mm	Top few cm	Morton & Miller (1973)
Paphies ventricosa	toheroa	>150mm	Top few cm –30cm	Hooker (unpubl. data) Stace (1991)
Paphies australis	pipi	≤50mm	Top 8–10cm	Morton & Miller (1973) Hooker (1995)
Paphies subtriangulata	tuatua	≤90mm	Top 8–10cm	Hooker (1995)

track the longshore movement of individuals. In this paper, we were able to use a metal detector to successfully monitor clams transplanted for small-scale experimental restoration. The metal detector used (Minelab Sovereign XS), has a detection range of approximately 20–30 cm below the surface for a 1 mm × 5 mm × 5 mm aluminum tag. The detection range is a function of tag size and burial depth of the target organism. The detection range can be improved by increasing the size of the tag, but this will ultimately be limited by the size and shape of the bivalve. Reported burial depths of some common New Zealand soft sediment bivalves suggest that aluminum tags may possibly be used for all these species (Table 3). Further experiments are needed to test for tag effects and the relocation efficiency of these other species, many of which live deeper in the sediment than *A. stutchburyi*. However, as reported here, the burial depth of *M. liliana* (≅ 20 cm) did not hamper the relocation of this species.

There was a low return rate for the whelks, as they rapidly move away from an area between tides, too quickly to be tracked. However the whelks that were relocated illustrated that the metal detector was sensitive enough to pinpoint a single tag. The recapture rate varied between the species tagged, being greatest for *C. virgata*. Therefore, while it may not be possible to follow movements of whelks over the long-term, the technique may still yield important information on movement patterns of other species.

Overall we believe that the simplicity, reliability, and versatility of this metal detection technique opens many new avenues for researchers in the area of soft-sediment ecology and restoration monitoring.

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ABSTRACTS OF PAPERS

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CONTENTS

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THE USE OF STIMULANTS AS AN AID TO WEAN FISHERY-CAUGHT BLACKFOOT ABALONE (*HALIOTIS IRIS*) TO ARTIFICIAL FOOD. **V. J. Allen, I. D. Marsden,** and **N. L. C. Ragg,** Zoology Department, University of Canterbury, Private Bag 4800, Christchurch, New Zealand.

Abalone use a combination of tactile and chemosensory feeding cues to detect suspended seaweed in their natural environment. However, in a commercial situation, adult abalone (*Haliotis iris*) caught for broodstock or pearling, show reluctance to start feeding on stationary artificial food and thus must be either induced to feed or lapse into a starvation phase that may last several weeks. Adult *H. Iris* (125 mm) were collected using SCUBA from Banks Peninsula and were held at Pendarves Abalone Farm Ltd (South Island, New Zealand). Multiple animals were offered a commercial pellet diet in a tank containing small quantities (0.03–0.05g dry weight per litre) of suspended seaweed particles (*Gracilaria spp*) acting as a tactile stimulant. Feeding and behavioural responses were monitored over 4 weeks in triplicate treatment tanks and compared to control tanks lacking the stimulant. Observations of behaviour over the first 10 hours showed that abalone held with and without stimulants spent the majority of their time alert (65.0 ± SE 7.6% and 75.0 ± 2.2% of time respectively). However, abalone held with stimulants also displayed typical receptive feeding posture (shell and foot raised) (21.0 ± 6.8% of time) and feeding on stimulants (2.1 ± 1.3%) whereas the control animals spent the remainder of their time either quiescent (15.0 ± 3.8%) or moving (6.0 ± 2.2%). Abalone fed stimulants in conjunction with artificial food took approximately 9 days to begin feeding on the artificial food. The abalone in the control experiment took 15 days to begin feeding on the artificial food and by day 21 their ingestion rate was only half that of the animals with stimulants (0.064 ± 0.005% and 0.127 ± 0.012% biomass ingested per day respectively). The collection and experimental procedures were repeated over four seasons to determine any seasonal effects on behaviour and feeding patterns. Implications for the maintenance of condition and survival of adult abalone in aquaculture will be discussed.

CHARACTERIZATION OF TRABECULAR CELLS IN THE GONADS OF *HALIOTIS ASININA*. **S. Apisawetakan,[1] M. Chanpoo,[1] C. Wanichanon,[1] V. Linthong,[1] M. Kruatrachue,[2] S. E. Upatham,[2,3] T. Pumthong,[4]** and **P. Sobhon,[1]** Departments of [1]Anatomy and [2]Biology, Faculty of Science, Mahidol University, Bangkok, Thailand 10400. [3]Department of Biology, Faculty of Science, Burapha University, Chonburi, Thailand, [4]Coastal Aquaculture Development Center, Department of Fishery, Ministry of Agriculture and Cooperatives, Prachuapkhirikhun, Thailand 77000.

Trabeculae are the connective tissue sheets that extend perpendicularly from capsules of both testis and ovary to make contact at their innermost ends with the loose connective tissue capsule of hepatopancreas. Thus they divide the gonads into small compartments, and each trabecula forms the axis for the spermatogenic or oogenic unit, from which maturing germ cells are generated. When studied using light and electron microscopes, each trabecula is shown to be composed of a central capillary, surrounded by a pack of smooth muscle cells and collagen fibers that are intermingled with small cells exhibited dense ellipsoid nuclei. Some of these cells are fibroblasts, while others are follicular or supporting cells that surround and may play a nurturing role for the developing germ cells. In addition, there are 3 types of granulated cells appearing in the trabecula connectives: the first type contains electron dense rugby-shaped granules with a diameter about 270 × 550 nm; the second type contains electron-dense spherical-shaped granules with diameters of about 165 nm; and the third type contains electron-lucent spherical-shaped granules with diameters of about 150 nm. These granulated cells may be the endocrine cells of the gonads, producing certain gonadotrophic factors yet to be identified.

THE EFFECT OF IRRADIANCE ON THE SURVIVAL AND GROWTH OF ABALONE POSTLARVAE *HALIOTIS FULGENS* FED WITH *NAVICULA INCERTA*. **C. Anguiano Beltrán, R. Searcy Bernal,** and **A. Esparza Hernández,** Instituto de Investigaciones Oceanológicas, Apartado Postal 453, 22860 Ensenada, Baja California, México.

Survival and growth of *Haliotis fulgens* postlarvae (11d old) were evaluated at four different irradiance levels, 6, 24, 47 and 75 microeinsteins·m^{-2}·s^{-1} (μE). Experimental vessels comprised 11 containers supplied with seawater flow and aeration. Four replicates per treatment were considered. Postlarvae were fed *Navicula incerta*, a benthic diatom. Survival and growth were evaluated. Shells were counted to determine mortality. To estimate shell size, video recorded images were processed by digital analysis. The highest survival (89.4% after 28d) and growth (37.1μm·d^{-1}) were found at 6 μE, whereas the lowest survival (3.5%) and growth (21.1μm·d^{-1}) occurred at 47 μE. These results suggest that abalone postlarvae have better survival and growth in the low light intensities under the particular conditions used in the present study.

DIET DEVELOPMENT AND EVALUATION FOR JUVENILE DONKEY'S EAR ABALONE, *HALIOTIS ASININA* LINN.: LIPID LEVELS. **M. N. Bautista Teruel** and **O. M. Millamena,** Aquaculture Department, Southeast Asian Fisheries Development Center, Tigbauan, Iloilo, Philippines 5021.

Juveniles of donkey's ear abalone, *Haliotis asinina* with mean initial weights of 0.71 ± 0.02g and shell lengths of 15.4 ± 0.04 mm were fed practical diets for 90 days. Diets contained 27% protein

with graded levels of lipid (0.25%, 1.5%, 3%, 4.5%) from a 1:1 ratio of tuna fish oil and soybean oil. Total lipid content of the diets were 2.2%, 4.69%, 7.7%, 10.7%. The fatty acid profile and proximate analyses of muscle meat of the test animals and published nutrient requirements of other *Haliotid* species were used as a basis for the practical diets developed. The diets were fed to abalone at 2–5% body weight once daily (1600h) for biological evaluation in terms of weight gain (WG), increase in shell length (SL), specific growth rate (SGR). The main effects of lipid levels on WG, SL, and SGR were significant at the 0.05 level with 5% total lipid significantly higher than all other lipid levels tested. The fatty acid composition of abalone juveniles reflected that of dietary lipids. Highly unsaturated fatty acids (HUFA) were noted to be incorporated more into the polar lipid fraction. Diet which contained the least amount of lipid had an increase in 16:0 and 16:1 fatty acids. The ratio of n3/n6 fatty acids was high in abalone fed diet containing 4.69% total lipid. Diet 2, containing 1.5% lipid from a 1:1 ratio of a tuna fish oil: soybean oil as lipid sources with a total lipid content of 4.69%, may be used as a basal diet for abalone juveniles.

COMPARATIVE GROWTH PERFORMANCE OF JUVENILE *HALIOTIS ROEI* FED ON ENRICHED *ULVA RIGIDA* AND VARIOUS ARTIFICIAL DIETS. **S. J. Boarder** Fremantle Maritime Centre, 1 Fleet St., Fremantle, Western Australia; and **M. Shpigel,** Israel Oceanographic and Limnological Research, National Center for Mariculture, P.O. Box 1212, Eilat, Israel.

The growth rates of juvenile *Haliotis roei* fed various artificial diets were compared with growth achieved from the consumption of inorganically enriched *Ulva rigida*. Juvenile abalone (20–40 mm S.L.) were collected from reef platforms off the Perth metropolitan area and assigned to one of seven different dietary treatments. All diets were fed *ad libitum* (3% b.w. day^{-1}) every second day and growth rates were quantified over a three month period. Specific growth rate (SGR), measured on a whole wet weight ($p < 0.01$) and shell length ($p < 0.01$) basis, indicates that abalone fed enriched *Ulva* grew at comparable rates to growth achieved from the best performing artificial diets. Reduced mortality rates in both the *Ulva* treatment and the best artificial diet also indicate a dietary advantage to general health through consumption of these diets. These results indicate that *Ulva* is a suitable feed for *H. roei*, providing comparable growth to that achieved from several commercially available diets. In contrast, other researchers have found *Ulva* to be a nutritionally poor food source for abalone when fed as a sole diet. In this study, enrichment of wild *U. rigida* increased the algal protein content from 11.4 ± 2% to 32.2 ± 1.5%, perhaps partially explaining the difference between this and other research. A comparison of survival under salinity stress for abalone from the different dietary treatments will also be discussed.

THE POTENTIAL RISK OF HARMFUL ALGAE TO ABALONE FARMING ON THE SOUTH COAST OF SOUTH AFRICA. **L. Botes,[1,2] G. C. Pitcher[2]** and **P. A. Cook,[1]** [1]Marine Biology Research Institute, Zoology Department, University of Cape Town, Rondebosch, 7701, Cape Town, South Africa; [2]Marine and Coastal Management, Private Bag X2, Rogge Bay, 8012, Cape Town, South Africa.

Toxic algal blooms are common world wide and pose a serious problem to the aquaculture and fishing industries. Of the dinoflagellates, species such as *Gymnodinium breve, Gymnodinium mikimotoi* and *Gyrodinium aureolum* are recognised fish-killers, implicated in various faunal mortalities. Toxic blooms of *G. cf. mikimotoi* were observed on the south coast of South Africa for the first time in 1988 and have subsequently been responsible for wild and farmed abalone (*Haliotis midae*) mortalities. Attempts to isolate an culture *G. cf. mikimotoi* revealed the presence of several gymnodinioid species on the south coast, namely: *G. pyrenoidosum, G. pulchellum, G. sanguineum, Gyrodinium cf. corsicum,* and *Lepidodinium viride.* Two other fish-killing species, namely: *Heterosigma akashiwo* and *Chatonella sp.* have also been isolated, as well as four more species that are common in this region namely, *Scripsiella trocoidea, Prorocentrum micans, Prorocentrum gracile,* and *Prorocentrum rostratum.* The species have been tested for toxicity by means of an *Artemia* bioassay (ARTOXKIT), a routinely-used method in marine and aquatic toxicology. A similar experimental procedure was used to assess the toxicity of each culture and its filtrate on both abalone larvae and spat (3 mm animals). Similar experiments were conducted on *Gyrodinium aureolum* (Isolation site: Norway; Obtained from: Department of Phycology, University of Copenhagen, Denmark) for comparative purposes. None of the species tested was toxic to *Artemia* larvae, but *G. sanguineum* was toxic to abalone larvae and spat, *G. pulchellum* was toxic to abalone larvae, and *G. aureolum* was toxic to abalone larvae and spat.

RIKETTSIALES-LIKE PROKARYOTES IN CULTURED AND NATURAL POPULATIONS OF THE RED ABALONE *HALIOTIS RUFESCENS*, BLUE ABALONE *HALIOTIS FULGENS*, AND THE YELLOW ABALONE *HALIOTIS CORRUGATA* FROM BAJA CALIFORNIA, MEXICO. **J. Cáceres Martínez, C. Álvarez Tinajero,** and **Y. Guerrero Rentería,** Centro de Investigación Científica y de Educatión Superior de Ensenada. Laboratorio de Biología y Patología de Moluscos. Apdo. Postal 2732, 2800 Ensenada Baja California; México, and **J. G. González Avilés,** Sociedad Cooperativa de Producción Pesquera. Pescadores Nacionales de Abulón, S. C. de R.L. Av. Ryerson 117, Ensenada, B.C. México.

Since 1995, Rikettsiales-like prokaryotes (RLP) in the black abalone, *Haliotis carcherodii,* have been considered as a presumptive causative agent of Withering Syndrome (WS) and they infect the ephitelial cells of the digestive tract of the host. Posterior field observations in California, USA, showed that other abalone species could present the characteristic symptoms of WS: shrunken appearance of foot muscle, retracted visceral tissues, and an inability to adhere tightly to the substrate. Recently, highly significant correlations between the presence of RLP and WS symptoms were found in farmed red abalone. In Baja California, México, no studies on the presence of RLP and WS in cultured or wild abalone populations have been carried out. To determine whether RLP are present in cultured and natural abalone populations in Baja California, México, a survey was carried out in WS symptomatic and non-symptomatic abalone obtained from commercial catches and aquaculture facilities in Isla de Cedros, Islas San Benitos and Bahía de Todos Santos B.C. It is important to note that this study was carried out during the occurrence of the "El Niño" phenomenon, between 1997 and 1998, when temperatures rose 2.5 °C above the normal range. Results showed the presence of RLP in cultured red and blue abalone. The prevalence of RLP in cultured red abalone was around 90%, infecting both WS symptomatic and non-symptomatic abalone. In cultured WS symptomatic blue abalone, its prevalence was 37.5%. These bacteria were also present in blue and yellow abalone from wild population, in which a prevalence of RLP of around 70% was recorded in both blue and yellow WS symptomatic and non-symptomatic abalone. However, the prevalence of RLP in blue abalone was greater (80.7%) than in yellow abalone (68.2%).

There was an increase in the prevalence of RLP and symptomatic WS abalone in samples taken in the middle and end of the "El Niño," when compared to the start of the phenomenon. The increase in RLP prevalence during the middle and end of "El Niño" when the effect of high temperature was evident (death of kelp beds, degradation in the sea floor) suggests that these conditions could interact as a synergic factor for the RLP presence and WS development. This observation supports the hypothesis that temperature-enhanced RLP infection plays a direct role in the etiology of WS. However, the presence of RLP and its association with WS symptoms remain confused and suggest that in addition to the role of temperature, the virulence of RLP could be affected by different strains or species of these bacteria. It is also probable that the susceptibility of different abalone species or individuals is different, or that an unknown condition-pathogen is also involved in WS development.

SYMBIONTS OF RED ABALONE *HALIOTIS RUFESCENS* FROM BAJA CALIFORNIA, MEXICO. **J. Cáceres Martínez** and **G. D. Tinoco Orta,** Centro de Investigación Científica y de Educación Superior de Ensenada. Laboratorio de Biología y Patología de Moluscos. Apdo. Postal 2732, 2800 Ensenada Baja California, México.

Although culture of commercial abalone started around 1973 in Baja California, no studies have been carried out on the symbionts of this species. In order to determine the symbionts of red abalone, *Haliotis rufescens,* a survey of healthy and moribund abalone from a cultured stock was carried out during "El Niño", 1997. Healthy abalone had a greater size (34 mm) than moribund abalone (25 mm). Macroscopical and microscopical analysis of the shell revealed the presence of two polychaetes belonging to the families Spionidae and Serpulidae, the former having a prevalence of about 10%, and the latter 100%. No serious ill-effects associated with the relationship between abalone and these worms were however observed. The histopathological evaluation showed the presence of the renal coccidia *Pseudoklossia haliotis,* its prevalence was 72% in moribund and 10% in healthy abalone. Rikettsiales-like prokaryotes were found in epithelia of the digestive tract of both groups with prevalence around 90%. Protozoan species were found in the branchial cavity of healthy abalone (17%) and moribund abalone (88%). A trend of high symbiotic prevalence and intensity in moribund rather than healthy abalone was recorded. Differences were not however statistically significant.

MICROHABITAT, DISTRIBUTION AND ABUNDANCE OF JUVENILES OF *HALIOTIS FULGENS* AND *H. CORRUGATA* IN BAHÍA TORTUGAS, MEXICO. **L. Carreón Palau, S. A. Guzmán del Próo, J. Belmar P., J. Carrillo L., R. Herrera F.,** and **A. Villa B.,** Laboratorio de Ecología, Departamento de Zoología, Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional. Prol. Carpio y Plan de Ayala s/n, México, D.F. 11340.

The distribution and abundance of juveniles of green and pink abalone (*Haliotis fulgens* and *Haliotis corrugata*) were studied at Bahía Tortugas, B. C. S, México, during the autumm and spring of

1996–98. Two sites with different depths, relief and wave exposure were surveyed at three different depths between 0.5 and 12 m deep; nine stations with 30 m^2 transects were sampled at each bank.

Four different habitats were recognized in each site: 1) sheltered, low bottom relief, 2) semiexposed, moderate bottom relief, 3) exposed, high bottom relief and 4) exposed sites, moderate bottom relief. Juveniles were not restricted to shallow waters, but were patchily distributed throughout the bank from 0.5 to 12 m depth, with higher densities being found in the shallow waters of the sheltered bank.

Emergent juveniles (3 to 13 mm length shell (l.s.)) were observed on small rocks, pebbles and flat stones not longer than 15 to 20 cm, always cryptic on the crustose coraline algae *Lithophyllum imitans*. Juveniles between 15 and 90 mm l. s. occurred under flat, rhombic or irregular shaped rocks, found primarily in imbricated arrangements.

Both species showed an aggregated distribution pattern, although this was not true of all environments. Green abalone juveniles were the more abundant species on both banks (0.15 ± 0.013 ab m^{-2}); pink abalone juveniles had a lower abundance (0.06 ± 0.01 ab m^{-2}). Significant seasonal changes in density were observed, primarily in green abalone, which declined in 1997 and recovered during spring 1998. Juveniles (20–90 mm) and adult forms share the habitat with briozoan, ascidians, chitons, anellids, gasteropods and echinoderms, while juveniles less than 13 mm share their habitat with tiny juveniles of *Fissurella*, chitons, *Crepipatella*, annelida and sea urchins. Size distributions suggest that both species have two cohorts, one from the summer/fall spawning season and another from the early spring season. The recruitment patterns of the species differ, suggesting that *H. fulgens* has an advantage under adverse climatic conditions such as during the 1997 El Niño event.

LOCALIZATION OF THE EGG-LAYING HORMONE (ELH) IN THE GONADS OF A TROPICAL ABALONE, *HALIOTIS ASININA* LINNAEUS. **M. Chanpoo,[1] S. Apisawetakan,[1] A. Thongkukiatkul,[3] C. Wanichanon,[1] V. Linthong,[1] M. Kruatrachuoe[2] S. E. Upatham,[2,3] T. Pumthong,[4] P. J. Hanna,[5] and P. Sobhon[1]** Departments of [1]Anatomy and [2]Biology, Faculty of Science, Mahidol University, Rama VI Rd, Bangkok, Thailand 10400, [3]Department of Biology, Faculty of Science, Burapha University, Chonburi, Thailand, [4]Coastal Aquaculture Development Center, Department of Fishery, Ministry of Agriculture and Cooperatives, Prachuapkhirikhun, Thailand 77000, [5]School of Biological & Chemical Sciences, Deakin University, Geelong, VIC 3217, Australia.

The connective tissue scaffold of the gonads of *Haliotis asinina* consists of the outer gonadal wall of fibro-muscular tissue, which forms a capsule-like structure. This capsule forms connective tissue trabeculae that partition the gonad into compartments. Each sheet of trabeculae contains a small capillary in the center, surrounded by a pack of smooth muscle cells and collagen fibers intermingled with small cells exhibiting dense ellipsoid nuclei. Some of these cells are fibroblasts, follicular cells and granulated cells that may synthesize hormones that induce spawning. Localization of the egg-laying hormone (ELH), which can induce the ovulation of the oocytes, was performed by immunofluorescence, immunoperoxidase and immunogold with silver enhancement techniques. Anti-ELH of *Haliotis rubra* exhibited strong staining in the trabeculae and the capsules, especially in the granulated cells within the trabeculae and the innercapsule, and the cytoplasm of oocytes stages 1 to 3, while the cytoplasm of oocytes stages 4 to 5 were only weakly stained.

THE PALLIAL ORGANS OF *HALIOTIS ASININA* LINNAEUS, 1758 (GASTROPODA : HALIOTIDAE) IN THAILAND. **Y. P. Chitramvong, M. Kruatrachue, E. S. Upatham, S. Singhakaew, and K. Parkpoomkamol,** Department of Biology, Faculty of Science, Mahidol University, Bangkok 10400, Thailand.

The pallial organs are composed of the gills, osphradium, hypobranchial gland, kidney, heart and rectum. The bipectinate paired gills are on the left and right sides of the mantle cavity. The left gill is smaller than the right one. They are light yellow in color. The paired osphradia are long, slender and lie anteriorly on each side of the mantle near the gills. Each has a long ridge with a central pleated groove. The cells are very tall and closely packed. The epithelial cells are ciliated cells. The paired hypobranchial glands are in the postero-dorsal view of the mantle near the rectum and the heart. They are usually composed of tall mucous cells and triangular ciliated cells. The right hypobranchial gland is better developed than the left one. The paired kidneys are glandular in structure. The left kidney is very well developed. It is bright orange-yellow in color and lies almost along the entire length of the mantle. It has a cuboidal epithelium and the secretory epithelium of the lumen of the kidney is folded into branched tubules. The heart is at the posterior end of the mantle connecting to the gills. It is composed of one ventricle and two auricles. The wall of the auricles contains very delicate lace-like strands of muscle and

fibrous tissue. The ventricle has thick opaque muscular walls. The rectum is attached to the left kidney. It is light yellow in color. Its length is about 1/3 of the length of the kidney and it has a ciliated columnar epithelium.

ISOLATION AND GROWTH OF SEVEN STRAINS OF BENTHIC DIATOMS, CULTURED UNDER TWO DIFFERENT LIGHT CONDITIONS. J. G. Correa Reyes, M. del Pilar Sánchez Saavedra, and **N. Flores Acevedo,** Aquaculture Department; Centro de Investigación Científica y de Educación Superior de Ensenada (C.I.C.E.S.E). Apartado Postal 2732, Ensenada, Baja California, México. C.P. 22800.

We have isolated seven strains of benthic diatoms from three different zones close to abalone seed farms in Baja California, México. We experimented using 10 replicates of non-axenic batch cultures of each strain in 250 ml Erlenmeyer flasks with 150 ml of Guillard & Rhyther's "f" medium. The cultured conditions had a salinity of 34 ± 1‰ and temperature of 22 ± 1 °C. Each strain was subjected to two different conditions of light (blue and white light) at the same irradiance (150 $\mu E\ m^{-2}\ s^{-1}$). Every second day for ten days we ultrasonicated the cultures and checked the cell concentrations, taking two flasks from each condition and strain (without replacement). The evaluation of cell concentration was measured by direct counts with a haemacytometer. Under these experimental conditions, we observed in all strains that the growth rate on the second or third day of culture had high values of duplication per day. We compared the growth rates of these strains with those of *Navicula incerta* (a strain used in some abalone farms in México), and found similar growth rates in some cases. For each strain, we used an analysis of covariance to compare whether there was any difference between strains grown under white and blue light and surprisingly found no significant differences. These results showed that some strains of benthic diatoms can be cultured under high light irradiances without any photoinhibition and they may have high growth rates and high cell concentrations ($\approx 5.0 \times 10^6$ cel^{-1} ml^{-1}).

CHEMICAL COMPOSITION OF EIGHT STRAINS OF BENTHIC DIATOMS, CULTURED UNDER TWO DIFFERENT LIGHT CONDITIONS. J. G. Correa Reyes, M. del Pilar Sánchez Saavedra, J. Arturo Simental Trinidad, and **N. Flores Acevedo,** Aquaculture Department, Centro de Investigación Científica y de Educatión, Superior de Ensenada (C.I.C.E.S.E.). Apartado Postal 2732, Ensenada, Baja California, México. C.P. 22800.

We carried out 10 replicates of non-axenic batch cultures of eight benthic diatom strains (two *Nitzchia* species, three *Amphora* species, two *Navicula* species and *Navicula incerta*) in 250 ml Erlenmeyer flasks with 150 ml of Guillard & Rhyther's "f" medium. The cultured conditions had salinities of 34 ± 1‰ and temperatures of 22 ± 1 °C. Each strain was cultured under two different continuous light conditions (blue and white light) at the same irradiance (150 $\mu E\ m^{-2}\ s^{-1}$). Biochemical cell compositions were determined for triplicate sets for each type of analysis, experimental condition and samples of each strain, on the sixth and tenth day of the culture. Biochemical composition (protein, lipids and carbohydrates) were determined using classic spectrophotometric methods; as expected, there were significant differences in biochemical composition between the eight diatoms strains. When the effect of light quality and diatom species was analysed using a two way ANOVA, there were no significant differences in biomass production. However, important differences in the biochemical composition of protein and lipids were found. The highest value of protein concentration was produced by *Nitzchia* sp. and the lowest value was for *Navicula* sp.

LOCATION OF EGG-LAYING HORMONE IN REPRODUCTIVE STRUCTURES AND NEURONS OF *HALIOTIS* USING ANTIBODIES RAISED AGAINST RECOMBINANT FUSION PROTEINS. S. Cummins and **P. J. Hanna,** School of Biological & Chemical Sciences, Deakin University, Geelong, VIC 3217, Australia; and **A. Thongkukiatkul,** Department of Biology, Burapha University, Bangsaen, Chonburi, Thailand.

Recombinant abalone egg-laying hormone was produced using a bacterial expression vector. This required TA cloning of a 108 bp abalone egg-laying hormone (aELH) gene sequence using, PCR of genomic DNA with primers incorporating restriction enzymes sites, into a pGEX-2T vector. Following transformation into *Escherichia coli,* a GST:aELH fusion peptide was produced and subsequently purified. This was used to immunise mice for production of polyclonal antibodies, and monoclonal antibodies, which were tested for specificity and reactivity using ELISA's, western blots and FITC assays. These antibodies are now being used in immunocytochemistry to determine expression of aELH during reproductive cycles and in which tissues.

PRELIMINARY TRIALS WITH "GREEN" ARTIFICIAL DIETS FOR JUVENILE ABALONE AND SEA URCHINS. C. Cuthbert and **G. Burnell,** Aquaculture Development Centre, Dept. of Zoology and Animal Ecology, UCC, Lee Maltings, Prospects Row, Cork, Ireland; and **J. Connolly,** Wm. Connolly & Sons Ltd., Red Mills, Goresbridge, Co. Kilkenny, Ireland.

This study was undertaken to evaluate and compare the effect of five artificial (formulated) diets (four Irish and one Australian diet) and a natural diet on the growth rates of juvenile abalone,

Haliotis discus hannai, and sea urchins, *Paracentrotus lividus*. Juvenile abalone with mean weight 0.21 (±0.07) g and sea urchins with mean weight of 1.22 (±0.03) g were fed artificial macroalgivore diets over approximately 150 days. The four Irish diets used contained a vegetable protein, instead of fishmeal protein, because the philosophy of the company was to develop an environmentally friendly diet. A seaweed (*Laminaria* spp) was included as a reference diet and a starved group served as a control. The diets were fed in excess every second day and all uneaten feed was removed prior to the next feed. Animals were maintained in 20l tanks (30–50 animals per tank) in a recirculation system. Temperature and salinity levels were monitored daily: mean values of 19 (±0.1) °C and 34.8 (±0.5) mg/l were recorded over summer months for these parameters. Ammonia, nitrate and nitrite levels in the systems were monitored weekly and more detailed chemical analysis was carried out monthly. No adverse build-up of organic wastes was noted. Biological evaluation was in terms of absolute weight gain (WG), increase in shell length (SL) or test diameter (TD) and specific growth rate (SGR). Physical evaluation was in terms of shell coloration (abalone only) and shell, test and spine (urchin) quality. Measurement of the above parameters took place during the first two weeks of each month, over the course of the study. Initial results show better growth performance in terms of WG, SL, TD and SGR for both species fed the five formulated diets, compared with those fed the natural diet. Initial poor shell margin quality of abalone on all formulated diets decreased over time as percentage growth between measurements decreased.

GROWTH AND SURVIVAL OF *HALIOTIS RUBRA* POST-LARVAE FEEDING ON DIFFERENT ALGAL SPECIES. S. Daume and **A. Krsinich,** Deakin University, School of Ecology and Environment, PO Box 423 Warrnambool, Victoria 3280 Australia; **S. Farrell** and **M. Gervis,** Southern Ocean Mariculture, RMB 2068, Port Fairy, Victoria 3284 Australia.

In previous experiments we showed that larvae of the abalone *Haliotis rubra* settle well on the encrusting green alga *Ulvella lens*. However, early growth on this alga is poor and settlement plates have to be inoculated with appropriate diatom species to complement feeding.

In this study, a flow-through system with large petri dishes was used to compare growth and survival of post-larvae feeding on different algal species. After settlement, small plastic sheets with *Ulvella lens* and recently settled post-larvae were transferred into replicated dishes and fed with different diatom species. The shell length of all post-larvae was measured before the trial and every week up to 2 months after settlement. Growth and survival rates were calculated to evaluate the success of the different diets. Faeces samples as well as grazed dishes were examined for broken diatom valves, to establish whether intracellular nutrients are available to post-larvae as a food source. The diatom species were chosen by their ability to attach strongly to the substrate and can therefore be used on vertical settlement plates in a nursery situation. Growth-rates were significantly higher on all treatments with additional diatom feed compared to the unfed control of the alga *Ulvella lens*. The best growth-rate was obtained with *Navicula* sp. During the first weeks after settlement, the majority of the diatom valves were not broken and were still alive in faeces samples of the post-larvae. After 3 weeks, most of the diatom valves in the samples were broken and intracellular nutrients became available to the post-larvae at this stage. This indicates that most of the nutrition for post-larvae during the first weeks after settlement must derive from extracellular material of the diatoms, which consists mainly of polysaccharides.

SETTLEMENT AND EARLY GROWTH OF THE ABALONE *HALIOTIS RUBRA* IN RESPONSE TO DIFFERENT ALGAL SPECIES. S. Daume and **A. Krisinich,** Deakin University, School of Ecology and Environment, PO Box 423 Warrnambool, Victoria 3280 Australia. **S. Farrell** and **M. Gervis,** Southern Ocean Mariculture (SOM), RMB 2068, Port Fairy, Victoria 3284 Australia.

Five benthic diatom species were isolated from settlement plates at SOM, Victoria, Australia and maintained in culture (*Navicula* sp. (small), *Navicula* sp. (large), *Nitzschia* sp., *Cocconeis* sp., *Amphora* sp.). The species were grown on plastic sheets and tested in settlement experiments with black-lip abalone (*Haliotis rubra*) larvae. Settlement was very low and varied between 1%–6%. When given a choice between the natural settlement substratum, the non-geniculate coralline red algae *Sporolithon durum* and single species diatom films, settlement was higher on *S. durum* than on any of the diatom films tested. High settlement of up to 36% was also achieved with germlings of the green encrusting alga *Ulvella lens*.

A flow-through system with large petri dishes was developed to compare growth and survival of post-larvae feeding on different algal species. One week after settlement, six post-larvae were transferred to each of four replicate dishes of each algal species. Four diatom and two macroalgal species were tested. The shell length of all post-larvae was measured before being transferred, and every week up to 11 weeks after settlement. Post-larvae grow

better on diatom films than on sheets with *Ulvella lens* or on pieces of *Sporolithon durum*. The best growth-rate was obtained with the large size *Navicula* sp. (39 ± 4 µm/d) and the lowest with the macroalga *Ulvella lens* (13 ± 3 µm/d). Overall the larger size *Navicula* sp. produced the largest juveniles (>3 mm shell length at the end of the 11 week trial) with the highest survival rate.

Ulvella lens and *Sporolithon durum* are both good settlement inducers but are not sufficient to support the rapid growth of young *Haliotis rubra* post-larvae. We suggest that plates with *U. lens* could be inoculated with diatom strains such as the large size *Navicula* sp. to ensure sufficient food for the growing post-larvae.

ARE ABALONE SHELL LAYERS DEPOSITED ANNUALLY? VALIDATION USING MANGANESE VITAL STAINING. **R. W. Day, G. P. Hawkes,** and **V. Gomelyuck** Zoology Department, The University of Melbourne, Parkville, 3052, Australia.

Management models of abalone fisheries would be greatly enhanced if we could age abalone with known confidence limits. Ageing of abalone using shell layers under the spire has received considerable attention since it was first proposed in Mexican abalone, but there has not been any rigorous validation of the method, or good estimate of ageing error. The manganese staining method we have developed here provides the first opportunity to validate properly the timing and frequency of these layers. Cathodoluminescent marks in recaptured tagged abalone identify subsequent shell growth over the release period. Validation trials were conducted at 3 Victorian and 3 Tasmanian sites for *Haliotis rubra*, and at 2 sites within South Australia for *H. laevigata*, by releasing 400–600 marked abalone at each site. Low recapture rates from Tasmanian sites and high mortality of *H. laevigata* from South Australia reduced the data available, but recaptures at periods up to one year showed great variability in the number of layers deposited subsequent to the mark. These results indicate that growth layers cannot be used to estimate individual abalone ages to within 1–2 years. However, monthly collections of *Haliotis rubra* from Port Philip Bay, Victoria, indicated that the timing of layer deposition within each year is very variable, and this may explain the variation seen. The monthly sampling also shows spire layers were most often deposited in March, after peak summer temperatures and before winter minima. This appears to contradict theories that rings are formed during non-growth periods in winter or during spawning events with increasing water temperature. Various sources of evidence suggest that the number of layers deposited per year in one animal may be remarkably consistent over longer periods. We found tentative direct evidence for this at two sites, Gabo Island and Port Phillip Bay, Victoria, where abalone were recaptured more than three years after release. However, the fact that only 5 of over 450 released abalone were recaptured at each of these sites after 3 years, shows that obtaining hard data for such long term age validation is limited by tag-and-release recapture.

THE PARASITE *TEREBRASABELLA HETEROUNCINATA* (POLYCHAETA) MANIPULATES SHELL SYNTHESIS IN *HALIOTIS RUFESCENS*. **R. Day,** Zoology Department, University of Melbourne, Parkville, Vic 3052, **C. Culver, A. Kuris, A. Belcher,** and **D. Morse,** Marine Science Institute, University of California at Santa Barbara, CA 93106, USA.

Terebrasabella heterouncinata, the sabellid polychaete that infests gastropod shells, has been introduced into California by abalone from South Africa. Dense infestations in *Haliotis rufescens* result in very distorted shells and reduced growth. The polychaete broods its young, and the juveniles crawl out of the burrow and into the space between the abalone mantle and the shell margin, where they secrete a tube. The effect of infection by juvenile sabellids on shell synthesis by the host was investigated using a manganese vital stain that allows identification of calcitic and aragonitic shell layers. Aragonitic nacre is deposited over the sabellid tubes by the host within 12 hours, about 4 to 8 times faster than normal aragonite synthesis. Simultaneously, extension of the shell through deposition of prismatic calcite along the margin, appears to be almost completely suppressed. The extent of this suppression may depend on the number of juveniles that have become established. After 2–3 days, a thick aragonite layer over the tubes extends to the shell edge. Small cuboid blocks of calcite are then deposited along the inside of the shell between the tubes, and the spaces between these blocks are later filled by further calcite deposition, forming a new broad growing edge oriented downwards, below the previous margin of the shell. These results explain the distorted shape and reduced growth of infected abalone. Control of shell synthesis is presumably mediated at least in part by the tube of the juvenile polychaete. Juveniles isolated in drops of seawater eventually produced tubes, which we stained with reagents. They apparently consist of muco-polysaccharides and proteins with both positive and negative groups on the surface. This differs markedly from the surface groups on the 'greensheet' protein synthesized by the abalone as a basement that guides shell formation.

QUANTIFYING THE PHYSICAL AND BIOLOGICAL ATTRIBUTES OF SUCCESSFUL OCEAN SEEDING SITES FOR FARM REARED JUVENILE ABALONE (*HALIOTIS MIDAE*). S. De Waal and **P. Cook**, Department of Zoology, University of Cape Town. Private Bag Rondebosch, 7701 Cape Town, South Africa.

In short term experiments carried out in Mac Dougalls Bay, on the northwest coast of South Africa, survival of seeded juvenile abalone (*Haliotis midae*) has been shown to be directly linked to both the size of the abalone at seeding and to the physical composition of the seeding site. The presence of the sea urchin (*Parenchinus angulosus*,) has been shown to play an insignificant role in terms of short term, up to two months, survival of juvenile *H. midae*. Within the context of selecting the right seeding site attributes, short term survival was shown to be up to a minimum average of 59% for animals of between 24 and 28 mm, compared to a minimum average of 24% for animals ranging between 12 and 16 mm. The fact that the presence of urchins played no significant role in the survival of juvenile abalone, ranging in size from 12 to 27 mm, changes the idea of what the characteristics of successful seeding sites might be. A positive correlation has been established between habitat consisting of stacked boulders of diameter less than 50 cm and abalone survival, with a negative correlation existing between the extent to which the area is exposed, when it offers no protection to juvenile abalone, and their survival.

POPULATION GENETICS OF THE YELLOW ABALONE, *HALIOTIS CORRUGATA*, IN CEDROS AND SAN BENITO ISLANDS. M. A. del Río Portilla, Centro de Investigación Científica y de Educación Superior de Ensenada Km 107 Carr. Tijuana-Ensenada, Ensenada, B. C. México A.P. 2732. Ensenada, México. 22800.

The yellow abalone, *Haliotis corrugata*, is the second species in production in Central Baja California. The Cooperative "Pescadores Nacionales de Abulón" has concessions for abalone exploitation off the Cedros and San Benito Islands. Abalone are mainly distributed in three large zones around these islands: a) the north (Punta Norte) and b) south (San Augustin) of Cedros Island and c) around the small islands of San Benito. The main goal of the present work was to characterize genetically the populations of yellow abalone in these three zones. Allozyme electrophoresis was carried out with six samples from two years in the three localities. The average number of allele per locus was 2.3 with a 67% polymorphism. Overall mean unbaised heterozygosity was 0.192 (range 0.151–0.251), which is similar to that of other abalone species, but a little higher to that of the blue abalone, *Haliotis fulgens*, from the same localities. Only one case out of 34 did not agree with the Hardy-Weinberg model and there was a tendency towards heterozygote excess, although this was not significant. A dendrogram with Nei's genetic distance was constructed using UPGMA analysis. The number of migrants per generation was less than unity. *Fst* statistics showed differentiation between localities. Therefore, these populations should be considered as independent populations for fishery management.

USE OF A SPREADSHEET MODEL TO INVESTIGATE THE DYNAMICS AND ECONOMICS OF A SEEDED ABALONE POPULATION. S. De Waal and **P. Cook**, Department of Zoology, University of Cape Town. Private Bag Rondebosch, 7701 Cape Town, South Africa.

Using a simple spreadsheet model, it is possible to investigate the dynamics of a discrete abalone population. The two variables used to drive the population are the percentage survival after the initial trauma of seeding, termed initial survival, and age differential survival, termed the survival regime. Of the two, initial survival is the variable that in practice plays the dominant role in determining the potential yield of any seeded population. There is experimental evidence that percentage survival in seeded abalone populations increases per age class; however, very little data exists for the species *Haliotis midae*. Seeding size plays a significant role in initial survival. The larger the animals, the higher the initial survival rate. Within the same experimental context, 13-mm animals showed a minimum survival rate of 24% while 26-mm animals showed a minimum rate of 56%, a non-linear increase in potential yield. The economic implications of both seeding scenarios are modeled. However, in order to be able to address the issue of potential yield on a commercial scale accurately, differential mortality amongst seeded populations of abalone needs to be researched.

PREFERRED TEMPERATURE AND CRITICAL THERMAL MAXIMA OF RED ABALONE *HALIOTIS RUFESCENS*. **F. Díaz, M. A. del Río Portilla, M. Aguilar, E. Sierra,** and **A. D. Re Araujo,** Centro de Investigación Científica y de Educación Superior de Ensenada Km 107 Carr. Tijuana-Ensenada, Ensenada, B.C. México, A.P. 2732, Ensenada, México, 22800.

Cultured red abalone *Haliotis rufescens* from Isla de Todos Santos Baja California, Mexico, were used in evaluations of preferred temperature and critical thermal maxima (CTM). Organism weights ranged from 12.7 to 26.0 g and from 4.7 to 6.0 cm in length. The red abalone locates its preferred temperature by the orthothermokinesis mechanism and the preferred temperature was 18.8 °C higher than optima previously reported for this species, higher than that of the South African abalones, but similar to the Australian abalones. The CTM of *H. rufescens* at 50% was 27.5 °C which is similar to other abalone species (*H. cracherodii, H. midae, H. rubra,* and *H. laevigata*).

CHARACTERISATION OF THE ENTERIC BACTERIA OF THE ABALONE *HALIOTIS MIDAE*, AND THEIR ROLE IN THE DIGESTION OF INGESTED SEAWEED. **K. Doeschate, B. M. Macey,** and **V. E. Coyne,** Department of Microbiology, University of Cape Town, Private Bag, Rondebosch, 7701, South Africa.

One of the challenges in farming abalone is to improve the growth rate of the animal. In many cases, the presence of bacteria within the digestive system has led to an improvement in the growth rate of the host animal. We have identified two bacterial isolates from the abalone digestive tract that exhibit increased levels of alginase (strain C4) and agarase activity (strain U5). Since maricultured abalone in South Africa are fed *Ecklonia maxima* and *Gracilaria gracilis*, which possess cell walls composed primarily of alginate and agar respectively, we are investigating whether these bacteria affect the nutrition of *H. midae*. The polysaccharolytic activity of *H. midae* was compared to that of the bacterial isolates in order to determine the extent to which each is able to hydrolyse alginate in *E. maxima* and agar in *G. gracilis*. The possibility that abalone use bacteria as a carbon and/or nitrogen source has been investigated by radiolabelling isolate C4 with [U-^{14}C] L-amino acids. Radiolabelled bacteria were orally introduced into abalone. Subsequently, various organs were dissected and ^{14}C-incorporation determined using a scintillation counter. Semi-artificial food, based on *E. maxima* and *G. gracilis* extracts, has been devised for use in growth rate studies in order to ascertain whether the abalone enteric bacterial isolates C4 and U5 influence the growth rate of the host animal. If abalone enteric bacteria are to be used as probiotics for farmed abalone, it is important to ensure that the bacteria colonise and persist in the host animal for a significant period of time. Thus, colonisation experiments are being conducted where C4 and U5, tagged with the luciferase enzyme, are tested for their ability to remain in the digestive tract of *H. midae*. Similarly, it is important to investigate whether the type of seaweed ingested by the host animal influences the composition of the microbiota colonising the gut of *H. Midae*. Thus, we have employed ribotyping as a tool for characterising the bacterial population colonising the abalone digestive tract. This data will allow monitoring of population changes in future experiments.

IDENTIFICATION OF EXPRESSED HSPs IN BLACKLIP ABALONE (*HALIOTIS RUBRA*) DURING HEAT AND SALINITY STRESSES. **B. Drew, Dean Miller, T. Toop,** and **P. Hanna,** School of Biological & Chemical Sciences, Deakin University, Geelong, VIC 3217, Australia.

Both prokaryotes and eukaryotes express a set of highly conserved proteins in response to external and internal stress. The stressors include tissue trauma, anoxia, heavy metal toxicity, infection, changed salinity, and the most characterised, heat shock. The result is an expression of stress proteins or heat shock proteins (HSP's) which lead to protection of protein integrity, and also to tolerance under continued heat stress conditions. The Australian blacklip abalone (*Haliotis rubra*) is found principally in southern coastal waters and also in estuarine/bay environments. Estuarine/bay environments have greater fluctuations in environmental conditions, especially those of salinity and water temperature, than are found along oceanic coasts. Abalone from estuarine/bay and oceanic coastal environments were subjected to either increased temperatures (2 °C/d, total of 10 °C) and lower salinity (75% seawater), in aerated tanks containing seawater and a local food source. Estuarine/bay abalone were less affected than the oceanic animals by temperature increase and also demonstrated the ability to regulate volumes 3h after the initial salinity shock. SDS-PAGE and Western blotting techniques, together with dot blots of total protein, using HSP70 specific antibodies, were used to visualize HSP70s in the foot muscle of the animals. Analysis of HSP70 mRNA expression, and cDNA library construction, are currently in progress to study the molecular basis of HSP response in abalone.

ONTOGENETIC CHANGES IN THE ACTIVITY OF MAIN DIGESTIVE ENZYMES DURING THE LARVAL AND JUVENILE STAGES OF ABALONE, *HALIOTIS DISCUS HANNI* INO. S. Du and **K. Mai,** Aquaculture Research Laboratory, Ocean University of Qingdao, Qingdao 266003, China.

This study was conducted to determine the ontogenetic changes in digestive enzyme activities during the larval and juvenile stages of abalone, *Haliotis discus hannai* Ino. Activities of protease, lipase, amylase, cellulase, laminarinase, carrageenase and alginase were analyzed in seven stages (before fertilization and 133 days after hatching). The results showed that activities of protease, lipase and carrageenase increased from fertilization to day 10 after hatching, then decreased; while activities of amylase, cellulase, laminarinase and alginase increased substantially and were fully developed at the 133 day. The changes in all these enzyme activities coincided with the development of the digestive system and the changes in natural diets of abalone.

RECOVERY AND GROWTH EFFECTS OF ANAESTHETIC AND MECHANICAL REMOVAL ON GREENLIP (*HALIOTIS LAEVIGATA*) AND BLACKLIP (*HALIOTIS RUBRA*) ABALONE. S. Edwards, C. Burke, S. Hindrum, and **D. Johns,** School of Applied Science, University of Tasmania, PO Box 1214, Launceston 7250 Australia.

Haliotis laevigata (39.7 ± 0.2 mm, 8.2 ± 0.1 g) and *Halitos rubra* (41.9 ± 0.1 mm, 11.3 ± 0.1 g) were acclimatised to conditions over 3–5 weeks (80 animals per 80 cm diameter fibreglass tank, flow-through sand-filtered seawater 17 °C, artificial diet ad-lib). Animals were then removed from the tanks using ethanol (3%), 2-phenoxyethanol (1 mL/L), benzocaine (100 ppm), clove oil (0.5–1.5 mL/L) and mechanical removal (metal spatula), measured, and returned to clean water for a further six weeks. At treatment, a sample of the animals was transferred to a multi-channel flow-through respirometer for analysis of oxygen uptake, which lasted at least 3 days. All treatments were duplicated for both species. One set (control) remained undisturbed from the beginning of the acclimation period to the end of the trial. Additional respirometry trials were conducted on the same cohorts for KCl (10 g/L), Aqui-S (50 ppm) and Tabasco (10 ml/L) that didn't fit in the growth trial.

Apart from first hour suppression (ethanol) or stimulation (clove oil & Aqui-S) of oxygen uptake, most agents showed shifted normal patterns of oxygen uptake settling to a normal value (~55 mg O_2.kg^{-1} h^{-1}) over 3–5 days. Increases in oxygen uptake were seen in first day averages for clove oil (156%), Aqui-S (154%) and KCl (127%). Mechanical removal gave first day suppression (50%) of oxygen usage, returning to normal with the evening activity cycle. Tabasco treated animals took longest to recover from a suppression of oxygen uptake. Benzocaine and KCl treated animals recovered most rapidly. There was no apparent recovery from clove oil in the time period studied. Growth trials showed healthy growth rates for control *H. laevigata* (116 ± 3 μm, 78 ± 4 mg per day) and all treatments indicated a suppression of growth rate as a result of removal from the tanks (48–83 μm, 19–70 mg per day).

For *H. rubra,* control growth rates were much lower (24 ± 1 μm/day) and weight gain was erratic (34 ± 10 mg/day). Nonetheless, lower growth rates (length 1.4–12.1 μm/day) were obtained for all treatments, while all but one treatment also had lower weight gain than control animals.

Animals subjected to clove oil had the lowest weight gain and this was the only treatment that resulted in significant mortalities.

APPLICATION OF MOLECULAR GENETICS TO THE UNDERSTANDING OF ABALONE POPULATION STRUCTURE—AUSTRALIAN AND SOUTH AFRICAN CASE STUDIES. N. G. Elliott,[1] B. Evans,[1,2] N. Conod,[2] J. Bartlett,[1] R. Officer,[3] and **N. Sweijd,[4]** [1]CRC for Aquaculture, CSIRO Marine Research, GPO Box 1538, Hobart, Tasmania 7001, Australia; [2]School of Zoology, University of Tasmania, Australia; [3]Tasmanian Aquaculture and Fisheries Institute, Hobart, Australia; [4]Department of Zoology, University of Cape Town, South Africa.

The structure of abalone populations is still poorly understood worldwide, and yet it is a major input to many management issues: wild fishery management, control of illegal fishing, selection of aquaculture broodstock and assessment of translocation or enhancement programs. Molecular genetic techniques have been advancing rapidly and there are now a number of techniques suitable for use in examination of population structure, each with relevant advantages and disadvantages. We report here on the application of two different types of molecular markers—mitochondrial RFLPs and nuclear microsatellites. The discriminatory power of the mitochondrial genome will be reported from studies conducted on both Australian (*Haliotis rubra*) and South African (*Haliotis midae*) abalone species. In addition, preliminary results will be presented from an on-going industry-funded microsatellite study on the major Australian blacklip abalone (*H. rubra*) population around the island of Tasmania. One aim of this two-year study is to apply a suite of polymorphic microsatellite loci to samples collected from 19 selected sites within the fishery, plus several sites along the southern Australian coastline. In addition, microsatellites developed from the blacklip abalone library have been tested for amplification and variation with other key Australian and overseas species to provide an initial suite of markers for use on populations of other species. The sampling plan and early results will be presented and discussed in context with other studies and our mitochondrial study.

IN VITRO DIGESTION OF CELLULOSE WITH STOMACH EXTRACTS FROM ABALONE (*HALIOTIS FULGENS*). A. Enríquez,[1] A. Shimada,[2] C. Vásquez,[3] and M. T. Viana,[4] [1]Facultad de Ciencias Marinas, Universidad Autónoma de Baja California, Ensenada, B. C., [2]Facultad de Estudios Superiores-Cuautitlán, Universidad Nacional Autónoma de México, Ajuchitlán, Qro, [3]Dirección Técnico Administrativa, Unidad de Zoológicos de la Ciudad de México, Chapultepec, D.F., [4]Instituto de Investigaciones Oceanológicas, Universidad Autónoma de Baja California, Ensenada, B.C. PO Box 450, Ensenada, B.C. 22860, México.

It had been reported that abalone are capable of digesting cellulose efficiently and that both bacterial and endogenous enzymes take part in the process. Stomach extracts from adult abalone collected in the vicinity of the "Emancipación" Fishery Cooperative were used to assess their capacity to degrade pure cellulose (alphacel) in the presence of a phosphate buffer (pH 5.5). The difference in glucose concentration before and after incubation was considered as a measure of cellulolytic activity (1 Unit mg^{-1} = relative increment of glucose after 2 h incubation). It appears that bacteria play an important role, as stomach extracts showed significantly decreased cellulolytic activity in the presence of antibiotics. A mixture of three antibiotics (chloramfenicol, cephalosporine and ampiciline) appeared to be the most effective growth inhibitor of stomach bacteria. Throughout a series in time, cellulolytic activity was detected in stomach extracts with antibiotics, even after 72 h incubation; the possible bacterial origin of the latter enzymes, is discussed.

THE USE OF MICROSATELLITE MARKERS FOR PARENTAGE ANALYSIS IN AUSTRALIAN BLACKLIP AND HYBRID ABALONE. B. Evans,[1,2] R. W. G. White,[2] and N. G. Elliott,[1] [1]CRC for Aquaculture, CSIRO Marine Research, GPO Box 1538, Hobart, Tasmania 7001, Australia, [2]School of Zoology, University of Tasmania, Hobart, Australia.

The understanding of mating processes in natural and cultured populations of abalone is required for a number of ecological and aquacultural questions. Mating and reproductive success are affected by behavioral, ecological and genetic aspects, all of which ultimately determine the transfer of genotypes from generation to generation. Parentage analysis can be used to either estimate the likely pair of parents for each progeny or to determine patterns of inheritance at the population level.

Relatively new DNA markers, microsatellites and AFLPs appear to offer the best potential for parentage analysis due to the level of variation available. Microsatellites are likely to be more useful as they are a dominant marker, whereas AFLPs show dominant/recessive inheritance, which would preclude identification of heterozygotes.

In order to establish a selective breeding program, known pedigreed family lines must be established and their progress compared. The infrastructure required to produce and maintain a large number of lines in isolation is high. The ability to identify the parents of all progeny from a mixed spawning event would alleviate the need for single pair crossing to produce the pedigree population.

Microsatellite DNA markers have been developed from a partial genomic library of the Australian Blacklip abalone, *Haliotis rubra*, and have been used to identify contributing broodstock in a Tasmanian abalone farm. The utility of the markers is demonstrated on known family lines and then tested on a batch of spawning animals. This technology is an essential part of the continued expansion of abalone culture worldwide, and the transfer of this technology for use on other species will also be discussed.

COMPENSATORY GROWTH AFTER INTERMITTENT FOOD DEPRIVATION AND REFEEDING IN THE DONKEY'S EAR ABALONE, *HALIOTIS ASININA* (LINNAEUS 1758). A. C. Fermin and S. Mae Buen, Southeast Asian Fisheries Development Center Aquaculture Department (SEAFDEC/AQD), Tigbauan 5021, Iloilo, Philippines.

Compensatory growth was determined after intermittent food deprivation and refeeding at different times during grow-out culture of abalone, *Haliotis asinina* in suspended mesh cages in flow-through tanks. In experiment 1, abalone were intermittently starved for 5 or 10 days and refed for the same duration during a 140-day period. Control groups were fed continuously on seaweed (*Gracilariopsis bailinae*). Fasted groups showed significantly lower feeding rates (DFR, 15–16% day^{-1}) than the control (22% day^{-1}) resulting in lower growth rates (DGR = 73–82 μm and 63–70 mg day^{-1}). Control groups had a DGR of 115 μm and 142 mg day^{-1}. When fed continuously over 60-days following the intermittent fasting and refeeding cycles, the fasted groups showed increased feeding rates (24–25% day^{-1}) resulting in improved daily growth rates (112–115 μm and 176–194 mg day^{-1}). The control group showed similar DFR (26% day^{-1}) but had lower

DGRs (42.4 μm and 71.2 mg day^{-1}). After 200 days, abalone in all treatments measured 46–48 mm SL and 25–28 g BW with no significant differences. The same animals were again subjected to a 5- or 10-day starvation with longer refeeding periods at 10 and 20 days respectively over 86 days, followed by continuous feeding for another 83 days. The control group fed continuously on seaweed. Results showed that abalone maintained high DGRs (90–93 μm and 169–183 mg day^{-1}) which were comparable with the control (89 μm and 183 mg day^{-1}). These findings were confirmed by another trial showing that abalone deprived of food for 10 or 20 days showed compensatory growth after refeeding for 20 or 40 days, respectively. Harvest size after 372 days ranged between 58–60 mm SL and 49–52 g BW with no significant differences among treatments. Likewise, percent survival (88 to 90%) was generally high and was similar for all treatments.

EFFECTS OF SEQUENTIAL FEEDING WITH SEAWEED AND ARTIFICIAL DIETS ON GROWTH DYNAMICS AND SURVIVAL DURING ON-GROWING OF ABALONE, *HALIOTIS ASININA* (LINNAEUS 1758). **A. C. Fermin, M. Bautista Teruel,** and **S. M. Buen,** Southeast Asian Fisheries Development Center Aquaculture Department (SEAFDEC/AQD), Tigbauan 5021, Iloilo, The Philippines.

The effects of sequential feeding with seaweed and artificial diet on the growth dynamics and survival rates of *Haliotis asinina* were determined as part of a feeding management protocol for on-growing of abalone in tanks. In Experiment 1, abalone juveniles (25 mm SL, 3 g BW) were initially fed either on seaweed (*Gracilariopsis bailinae*) or fish meal and soybean meal-based artificial diet (27% crude protein) during the first 12 weeks, and then interchanged with opposite feeds for the final 12 weeks. Control groups were fed either seaweed alone or artificial feed alone throughout the duration of culture. In Experiment 2, juveniles (33 mm SL, 9 g BW) were fed artificial feeds (27% crude protein) for 60 or 90 days, after which seaweed was given for the rest of the 150-day culture period. Control groups were fed seaweed only.

Results of Experiment 1 showed that groups that fed on seaweed during the first 90 days had significantly higher daily growth rates (179–180 μm and 84–89 mg day^{-1}) than those fed with artificial diets (–6.25–29 μm and 23–38 mg day^{-1}). When feeds were interchanged, animals that were re-fed on seaweed showed the highest growth rates (220 μm and 175 mg day^{-1}) after 70 days. Abalone that were re-fed on artificial diets showed the lowest growth rates, comparable to the artificial diet-control. However, percent survival (79%) was significantly higher in the seaweed-control than in the rest of the treatments (range:33–53%). In Experiment 2, abalone fed artificial diets at 60- and 90-day rearing periods showed growth depression as indicated by significantly lower growth rates (24–59 μm and 14–16 mg day^{-1}) than the control (94 μm and 118 mg day^{-1}). However, animals exhibited compensatory growth after resumption of seaweed feeding, and fed similarly to the control (34 μm and 126 mg day^{-1}). At the end of a 150-day culture period, abalone fed artificial diets at shorter duration (60 days) had significantly higher growth rates (117 μm and 207 mg day^{-1}) than the control. Percent survival was generally high (93–97%) with no significant differences among treatments. Abalone exhibited growth depression when fed artificial diets. However, animals showed compensatory growth upon resumption of feeding on fresh seaweeds. Results of both experiments indicated that fresh seaweed using *G. bailinae* proved to be advantageous and more convenient to use than artificial feeds.

EXAMINATION OF THE GEOGRAPHIC DISTRIBUTION OF A *RICKETTSIA*-LIKE PROKARYOTE IN RED ABALONE, *HALIOTIS RUFESCENS*, IN NORTHERN CALIFORNIA. **C. A. Finley**[1,2] and **C. S. Friedman,**[2] [1]Humboldt State University, Fisheries Department, Arcata, CA 95521-8299, [2]California Department of Fish and Game and Bodega Marine Laboratory, P.O. Box 247, Bodega Bay, CA 94923 USA.

Withering syndrome (WS) is a chronic wasting disease responsible for mass mortalities in wild populations of black abalone, *Haliotis cracherodii*, in southern California. A recently discovered *Rickettsia*-like prokaryote (RLP) has been identified as the causative agent of the disease. The RLP has been positively identified in both wild populations of black abalone with WS and cultured red abalone, *H. rufescens*, which displays signs characteristic of WS. Concern has risen that the culture facilities, as well as outplantings that took place prior to the identification of the RLP, may have served as vectors for the disease into northern California red abalone populations, the only remaining healthy populations of abalone in the State. In this study, five point source locations and four reference locations north of Carmel (where the RLP was previously observed) were chosen using a stratified random design. Strata were defined either by their proximity to an out-plant location or an abalone facility (both point sources) or as reference

locations (found between point sources). At each site 60 animals were randomly collected to achieve a 5% detection level. Animals were examined for the presence of WS and the RLP, and the shells were also examined for the presence of an exotic sabellid, *Terebrasabella heterouncinata*, another aquacultural pest of concern. Preliminary data show the RLP has spread north to San Francisco (at both point and reference sites). The RLP has not been observed in a point source and a reference site examined north of San Francisco. In addition, no sabellids have been identified in any of these samples. At this point, the distribution of the RLP does not appear to have been influenced by either culture facilities or outplantings, but several sites have yet to be examined.

LIFE HISTORY OF AN EXOTIC SABELLID POLYCHAETE, *TEREBRASABELLA HETEROUNCINATA*: INFLUENCE OF TEMPERATURE AND FERTILIZATION STRATEGY. **C. A. Finley,[1,2] C. S. Friedman,[2] and T. J. Mulligan,[1]** [1]Humboldt State University, Arcata, CA 95521-8299, [2]California Department of Fish and Game and Bodega Marine Laboratory, P.O. Box 247, Bodega Bay, CA 94923 USA.

The California abalone aquaculture industry has been struggling to rid itself of an exotic sabellid, *Terebrasabella heterouncinata*, following its accidental introduction from South Africa in the late 1980s. Intensive culture conditions and periodically elevated seawater temperatures, associated with El Niño events, have created conditions in which rapid spread and intensive infestations have occurred in culture facilities. These observations have raised concerns over the potential threat that the sabellid may pose to native invertebrate populations throughout California, following an accidental introduction from an aquaculture facility. California's seawater temperatures range from a low of 8 °C to 21 °C and exceed these averages during El Niño years. A need has arisen to improve understanding of the life history of this sabellid, including generation time and whether it is capable of self-fertilization. In the present study, uninfested red abalone, *Haliotis rufescens*, were exposed over a 24 hr period to heavily sabellid-infested abalone at 10, 16, and 20 °C. The larvae were subsequently observed as they developed to specific life stages: initiation of feeding, sexual maturation and the completion of their life history or the production of a motile, infestive, larva. Approximately 50% of the sabellids examined had developed the ability to feed by day 6, 5, and 4, and became sexually mature by day 83, 68, and 49 for 10, 16, and 22 °C, respectively. Preliminary results indicated that 50% of the sabellids had produced larvae by day 111 at 22 °C. In a separate study, uninfested abalone were exposed as above, and abalone with single infestations were held in individual containers at 18 °C (single host and sabellid per container). This first, parental generation was held in isolation until individuals self-fertilized to produce F_1 larvae. The F_1 larvae were allowed to infest new abalone (single sabellid per host) and were then isolated as above. We subsequently observed second-generation, F_2 larvae. This research demonstrates that the life history and generation time of *T. heterouncinata* are highly temperature dependent and that its products of self-fertilization are fully functional organisms.

"CANDIDATUS *XENOHALIOTIS CALIFORNIENSIS*," A NEWLY DESCRIBED BACTERIAL PATHOGEN AND ETIOLOGICAL AGENT OF WITHERING SYNDROME FOUND IN ABALONE, *HALIOTIS* SPP., ALONG THE WEST COAST OF NORTH AMERICA. **C. S. Friedman,[1,2] K. B. Andree,[2] T. T. Robbins,[2] J. D. Shields,[4] J. D. Moore,[2] K. Beauchamp,[2] and R. P. Hedrick[2]** [1]California Department of Fish & Game, Bodega Marine Laboratory, P.O. Box 247, Bodega Bay, California 94923, [2]Department of Medicine & Epidemiology, School of Veterinary Medicine, University of California, Davis, California 95616, [3]Virginia Institute of Marine Science, Gloucester Point, VA 23062.

Withering syndrome (WS) is a fatal disease affecting wild and cultured abalone, *Haliotis* spp., which inhabit the west coast of North America. A previously undescribed bacterium observed in abalone with WS has recently been identified as a member of the family Rickettsiaceae in the order Rickettsiales. Using a combination of morphological, serological, life history and genomic (16S rDNA) characterization, we have identified this bacterium as a new genus and species. Due to the inability to culture the bacterium we propose the provisional status of "Candidatus *Xenohaliotis californiensis*." The Gram negative, obligate intracellular, pleomorphic bacterium is found within membrane-bound vacuoles in the cytoplasm of abalone gastrointestinal epithelial cells. The bacterium is not cultivable on synthetic media or in fish cell lines (e.g., CHSE-214) and may be controlled by tetracyclines (oxytetracycline) but not by chloramphenicol, clarithromycin, or sarafloxicin. Phylogenetic analysis based on the 16S rDNA of "Candidatus *Xenohaliotis californiensis*" places it in a subclass of the class *Proteobacteria*. The bacterium can be detected in tissue squashes stained with propidium iodide, microscopic examination of stained tissue sections, PCR or *in situ* hybridization. "Candidatus *Xenohaliotis californiensis*" can be differentiated from other closely related *Proteobacteria* by its unique 16S rDNA sequence. We tested the hypothesis that this bacterium is the etiological agent of WS in two long term clinical trials using oxytetracycline as the therapeutant. In two separate trials asymptomatic red and, in a separate trial, black abalone +/−WS administered a series of sham injections (3% saline as controls) or oxytetracycline injections (21 mg/kg Liquamycin LA-200 as the experimental treatment) over a 9 wk period. Survival and feeding rates were monitored for 6 mo. Both survival and feeding rates were higher in treated abalone relative to control animals ($p < 0.001$, $p < 0.023$ for red and black abalone, respectively). All red abalone and ~50% of the black abalone that received the therapeutant survived, while ~40% of the red and 100% of the black abalone controls died during this time. These studies indicate that WS is caused by "Candidatus *Xenohaliotis californiensis*" and that losses can be minimized by administration of oxytetracycline.

THE CELLULAR IMMUNE RESPONSE OF BLACK ABALONE, *HALIOTIS CRACHERODII* LEACH, WITH AND WITHOUT WITHERING SYNDROME. **C. S. Friedman,[1] T. Robbins,[1] J. L. Jacobsen,[2]** and **J. D. Shield,[3]** [1]California Department of Fish and Game and Department of Medicine and Epidemiology, Bodega Marine Laboratory, P.O. Box 247, Bodega Bay, CA 94923, [2]Bodega Marine Laboratory, P. O. Box 247, Bodega Bay, CA 94923, [3]Virginia Institute of Marine Science, Gloucester Pt., VA 23062.

Withering syndrome (WS) is a chronic disease that has resulted in dramatic declines in black abalone abundances along the southern and central California coast. A *Rickettsia*-like procaryote has recently been identified as the etiological agent. We hypothesized that the nonspecific immunity function of abalone hemocytes may be affected by WS and that these changes may serve as early indicators of disease. We examined the chemotactic, phagocytic and chemiluminescent abilities of hemocytes from abalone with and without WS. Although hemocytes from abalone with WS were more chemotactically active than those from asymptomatic abalone ($n = 35$, $p < 0.01$), they were compromised in other key functions. Hemocytes from diseased abalone were less able to engulf foreign particles ($n = 59$, $p < 0.01$), engulfed fewer particles ($n = 52$, $p = 0.00$), and produced a reduced respiratory burst ($n = 26$, $p = 0.00$) relative to those from asymptomatic abalone. The immune capability of the hemocytes correlated with the degree of WS. Thus, hemocytes from abalone with WS may be more chemotactically active as a result of degeneration of the digestive gland and utilization of the foot muscle as an energy source. However, the capability of these stimulated cells to engulf and destroy foreign particles appears to be compromised and may contribute to mortality associated with this disease.

EVALUATION OF ON-SHORE AND SEA-BASED CULTURE SYSTEMS FOR ROE'S ABALONE (*HALIOTIS ROEI*) IN WESTERN AUSTRALIA. **K. J. Friedman, G. Maguire,** and **K. O. Hahn,** Fisheries WA. Western Australian Marine Research Laboratories, PO Box 20, North Beach, Perth, Western Australia 6020.

Fisheries Western Australia is conducting research to evaluate the potential of local abalone species for culture. Although the state has a healthy wild abalone fishery, there is great interest in producing abalone through aquaculture techniques. To date, abalone aquaculture in Australia has concentrated on greenlip (*Haliotis laevigata*), blacklip (*Haliotis rubra*) and hybrid abalone. Unfortunately, these animals are coldwater species and unsuitable for the warm water along the western coast of Australia. The Roe's abalone (*Haliotis roei*), fished commercially and found in great abundance, offers an opportunity to culture abalone in the warmer waters of Western Australia. Fisheries WA is conducting a grow-out study to determine the suitability of Roe's abalone for culture. The study is evaluating three culture systems-Australian raceways, California round settlement tanks, and sea-based barrels-for grow-out of juvenile Roe's abalone. These culture systems are being used at several sites within WA to determine whether there are differences in growth and survival by location. Greenlip abalone (*H. laevigata*) are being reared alongside the Roe's abalone to provide a direct comparison of growth and survival. Variables being investigated in replicated land-based tanks include tank design, location (over the range 29–35 °S), current speed, turbulence and shading.

SEEDING COMPETENT CULTURED LARVAE OF THE BLUE ABALONE *HALIOTIS FULGENS* INTO SOME WILD STOCKS OFF THE ISLAND OF CEDROS BAJA CALIFORNIA, MEXICO. **O. J. G. González Avilés,** S.C.P.P., P.N.A., Av. Ryerson 117, Ensenada, B. C. México.

Due to high poaching pressure on natural abalone populations and with the additional negative effect of "El Niño" (1997) abalone stock density has declined in some areas. In our areas and given our resources, larval stock enhancement is more viable than abalone juvenile seeding. Therefore, for several years, our cooperative has cultured viable larvae for stock enhancement using wild broodstock collected directly from natural stocks. In this work we describe the larval releasing process. A semiautomatic diving "hooka" system is used for seeding. Competent larvae are placed *in situ* from no mixed stocks to avoid genetic exchange. Data from five years of larval releasing is shown and analysed.

WORLD ABALONE SUPPLY, MARKETS & PRICING. **H. Roy** and **Gordon N. Qing,** China and Taiwan, **N. Ujki,** Japan, **R. Fields,** USA, **R. Flores,** Mexico, **A. Ziomi,** South America, **M. Tokley,** Australia/Tasmania, **R. Roberts,** New Zealand, **P. Cook** and **A. du Plessis,** South Africa, **G. Burnell,** Ireland and Europe.

This session will comprise a country by country overview of both wild fisheries and cultured abalone. Each speaker has gathered important tonnage and pricing data along with details of species caught or grown, cultivation methods, feed information and disease implications, and introductions which may include past and present trends as well as locations of particular interest in each country. Species discussion may include locations and in the case of cultured abalone, hybrids. Types of cultivation may include a discussion of various systems including both land and ocean cultivation. Feeding discussions will include manufactured food as well as available or cultured algae. Pathology comments will include an overview of past and current problems and may include order of magnitude impact of each disease.

WORLD ABALONE SUPPLY, MARKETS AND PRICING FROM HISTORICAL, CURRENT AND FUTURE PROSPECTIVES. H. R. Gordon, Fishtech Inc, Box 6886 San Rafael, California 94903.

The world supply of wild fisheries catch and cultured abalone production are considered with implications of the past, present and future. Much of the data available in recent years from various government sources and even FAO have often been inadvertently misleading, as quantities reporting is developed using differing and sometimes conflicting base lines. Catch and production data in some countries have been either nonexistent or with substantial gaps in reporting. Reporting has often combined a number of dissimilar abalone products (fresh in shell, frozen meat, canned, dried etc) with misleading results. As a further complication, export numbers in some cases inadvertently include illegal catch abalone. Reporting from the cultured or farmed abalone "world" has been limited and much of the available information has been reported using differing standards. Surprising numbers result from this effort to standardize the production and export information for both the commercial catch and cultured product. Comparative charts and order of magnitudes have been developed. The decline in total world supply from the 1980's is given an order of magnitude in relation to poaching, pollution, pathology, predators and climate. Discussion will include factors affecting abalone prices worldwide, a review of market form (live, fresh, frozen, canned, dried) and how pricing is affected by processing and packaging as well as economic conditions, followed by a review of suggestions for value added abalone product. The implications of Asian traditions are also described and explained. Ethnic preparation and cooking methods are summarized along with their impact on world market prices.

POST HARVEST WEIGHT LOSS HAS IMPORTANT IMPLICATIONS FOR ABALONE QUOTA MANAGEMENT. H. K. Gorfine, Marine & Freshwater Resources Institute, PO Box 114, Queenscliff, VIC, Australia.

Regulations forming part of the abalone quota management system in Victoria, Australia, require that abalone processors notify fisheries authorities of quantities of abalone consigned from commercial divers within 25 hours of landing. The regulations also require that the abalone are to be landed whole in the shell, and transported and stored in sealed bins until one half hour after official notification. Whilst the bins cannot be opened prior to notification, the 25 hour limit for notification provides a window of opportunity to make potential gains through weight loss in those abalone destined for canning. This arises because notified weights, rather than weights estimated upon landing, are used to decrement the quota allocations of individual divers.

I investigated the potential for post-harvest weight loss by subjecting abalone to three experimental treatments selected to simulate a range of possible transport and storage regimes. My results demonstrated that substantial weight loss can occur in whole abalone during both ambient and refrigerated storage. Loss of weight in abalone during storage results from the release of water and body fluid associated with physiological responses to hypoxic stress caused by exposure to air. In Victoria, abut 60% of the landed catch is used to produce canned product. At present, weight losses during storage are not accounted for in setting the Total Allowable Catch and divers supplying abalone for canning have to harvest more abalone to achieve their quotas than those supplying abalone for live export. Losses in weight of 10–20% observed during this study equate to 350,000–700,000 more abalone harvested than if beach weights were deducted from quotas. These additional quantities of abalone harvested may exceed desired fishing mortalities for long-term population sustainability.

A BEHAVIOURAL RATHER THAN RESOURCE-FOCUSED APPROACH MAY BE NEEDED TO ENSURE SUSTAINABILITY OF QUOTA MANAGED ABALONE FISHERIES. H. K. Gorfine and **C. D. Dixon,** Marine & Freshwater Resources Institute, PO Box 114, Queenscliff, VIC, Australia.

The Victorian blacklip abalone fishery is Australia's second largest fishery and accounts for about one eighth of reported global abalone catch. Most indicators of stock status for this fishery are favorable, with relatively high daily catch expectations (about 500 kg) among divers. The fishery is subdivided into three management zones, each spanning several hundreds of kilometers of coast, and within each zone divers are restricted to harvesting an equal share of the total allowable catch. During 1998 we initiated an on-board observer program to gain a better understanding of spatial and temporal patterns in catch and effort. Although average CPUE has been increasing, there has also been significant spatial contraction of the fishing grounds away from reefs of low productivity, consistent with the effects of quota introduction during 1988/89 and with subsequent serial depletion. It is this shift away from reefs of low productivity rather than an increase in abalone abundance that is responsible for the trend in CPUE. From our on-board observations, divers do not operate in an area if they believe that they will not meet their daily catch expectations; they have a relatively high catch rate threshold for deciding when to shift to another reef. Catch rates per bag of abalone are several times higher than the daily reported CPUE rates, but vary substantially. We conclude that under quota management, spatial re-

allocation of effort and incentives to maintain high catch rate thresholds for cessation of fishing at the reef scale in an environment where competition for prime fishing grounds is reduced through restricted entry have been the keys to sustainability of the Victorian fishery during the past 35 years. However, contemporary changes in the fishery, such as reductions in the number of divers who own access entitlements, may lead to unfavourable fishing behaviour patterns among divers. Managers need to be aware that regulations affect diver behaviour and that, despite increased interest in resource manipulation through restoration and sea ranching, it is the diver and not the resource that is managed. Our studies shows that it is important to focus on identifying and promoting behaviour among divers that is desirable in terms of sustainable production within the context of contemporary management strategies.

TRIGGERS AND TARGETS: WHAT ARE WE AIMING FOR WITH ABALONE FISHERIES MODELS? H. K. Gorfine, B. L. Taylor, and **T. I. Walker,** Marine & Freshwater Resources Institute, P.O. Box 114, Queenscliff, VIC, Australia.

A variety of quantitative measures have been applied as reference points in the management of Australian abalone fisheries. In New South Wales, changes in legal-sized and mature biomass will trigger management responses; in South Australia, catch rates, size composition and abundance indices provide target reference points and in Tasmania, catch rates are used to provide triggers for management decisions. However, Victoria and Western Australia have yet to determine their reference points for abalone stock assessment. Victoria has been developing length-based fisheries models similar to those applied in NSW, and is now confronted with the necessity of converting model outputs into decision-making criteria. A Victorian fishery management plan is also under development in which reference points will be specified within a risk-based matrix of catch control rules for TAC adjustment. Recent biodiversity conservation legislation, compelling fisheries management agencies in Australia to demonstrate that export fisheries managed under their jurisdictions are ecologically sustainable, has increased the urgency to establish these reference points. The application of this legislation draws upon the 'Principles and Criteria for Sustainable Fishing' of the Marine Stewardship Council in London. We considered a range of alternative measures for reference points that may be useful as triggers and targets applied in a stochastic framework for management decisions. Although not a modeling output, one of the more consistent signals of localised depletion in the Victorian fishery relates to spatial allocation of effort at the scale of reef complexes. Reductions in annual effort applied to a particular reef system invariably precede significant decreases in abundance indices with typically large coefficients of variation and catch rates characterised by hyperstability. Victorian abalone divers have high daily catch expectations and allocate their effort accordingly. Empirical reference points such as effort allocations provide utility for fishery management, and can be readily assimilated and adopted by industry. Consequently, we conclude that maintenance of reef-scale effort allocation and daily catch expectations should form part of a suite of fishery performance indicators and target criteria related to modelling outputs for the Victorian blacklip abalone fishery.

VARIATION IN MINERALOGY IN THE NEW ZEALAND BLACKFOOT ABALONE *HALIOTIS IRIS* SHELL. Blair Gray, Department of Marine Science, University of Otago, P.O. Box 56, Dunedin, New Zealand.

The blackfoot paua (abalone) *Haliotis iris* is the most common of the three species of abalone occurring in New Zealand, and has been commercially fished for its meat and shell for many decades. In more recent times, there has been increasing interest in pearl production using the paua shell.

The shells of the majority of gastropod species consist of aragonite, and only a few species belonging to 13 families, utilise calcite as part or the whole of the shell. *Haliotis iris* has the ability to control the growth of two crystal polymorphs of calcium carbonate ($CaCO_3$). These biologically formed polymorphs, calcite and aragonite, have the same chemistry but vary in their crystallographic arrangement, giving them different physical and chemical properties. The location and thickness of these mineral layers was examined in *Haliotis iris*, using both Feigl's Solution and Scanning Electron Microscopy (SEM). These techniques confirmed the presence of an outer calcitic layer and inner aragonitic layers. These are separated by both calcified and non-calcified organic layers running longitudinally through the shell.

Many of the classical studies on the mineralogy of Molluscs have only used a small sample size to assess the mineralogy of a species. Even fewer studies have examined the variation within the shells of individuals. This study examined the varying amounts of calcite and aragonite within individual shells and within populations using X-ray diffraction (XRD). It was found that there is a significant difference both within individual shells ($p < 0.01$) and individuals within a population ($p < 0.01$). Within a population, the variation in mineralogy may be as high as 40–93% aragonite. This finding may have important implications for the classification of mineralogy for not only Haliotids, but Molluscs in general.

This paper will also make an attempt to correlate the variation in mineralogy between populations and environmental parameters such as wave exposure, seawater temperature, and salinity, as well as to estimate the effects of erosion and biotic interactions e.g., epiphyte growth and shell boring.

GENETIC VARIABILITY OF THE BLUE ABALONE *HALIOTIS FULGENS* IN THE WEST COAST OF BAJA CALIFORNIA, MEXICO. **José L. Gutiérrez González,[1] Ana M. Ibarra,[2]** and **Miguel A. del Río Portilla,[3]** [1]Universidad Autónoma de Baja California Sur/Centro Regional de Investigaciones Pesqueras La Paz, [2]Centro de Investigación Biológicas del Noroeste. A.P. 128. La Paz B.C.S. 23000 México, [3]Centro de Investigación Científica y de Educación Superior de Ensenada Km 107 Carr. Tijuana-Ensenada, Ensenada, B.C. México A.P. 2732. Ensenada, México 22800.

Even though on the west coast of Baja California Peninsula there are five commercially exploited species of abalone, of which *Haliotis fulgens* (blue abalone) produces more than 50% of the catch, little research has been done on population genetics. Thus, the main goal of the present work was to characterize genetically the blue abalone populations in Baja California. The Peninsula is divided into four abalone exploitation zones. Therefore, an area with high abalone density from each zone was sampled, as follows: a) Isla de Cedros, zone I; b) Bahia Tortugas, zone II; c) Bahia Asuncion, zone III and d) Isla Magdalena, zone IV. Allozyme electrophoresis was carried out for a total of 377 organisms at 16 loci of which 11 were polymorphic. The proportions of polymorphic loci were 37.5% in Bahia Asuncion and 43.7% for the other three populations. Twenty-five cases did not adjust significantly to the Hardy-Weinberg (H-W) model, and all of them had heterozygote deficiencies. It was considered that the four populations as a whole were not in H-W, having high heterozygote deficiency. Mean unbiased heterozygosity ranged from 0.173 tp 0.197, where Bahia Tortugas had the smallest value. Cluster analysis first joined Bahia Asuncion and Isla Magdalena as one group, then linked them with Isla de Cedros and finally with Bahia Tortugas. Most of the F values were positive and the mean Fst was 0.022 showing a low genetic diversity.

DISPERSION POTENTIAL OF REPRODUCTIVE PRODUCTS AND LARVAL STAGES OF ABALONE (*HALIOTIS* SPP.; MOLLUSCA:GASTROPODA) IN RELATION TO THE HYDRODYNAMICS OF BAHÍA TORTUGAS, MEXICO. **Sergio A. Guzmán del Próo, Felipe Salinas, Oleg Zaytsev, Jorge Belmar Pérez,** and **Jorge Carrillo Laguna,** Laboratorio de Ecología, Departmento de Zoología, Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional. Prol. Carpio y Plan de Ayala s/n, México, D. F. 11340.

Field observation of currents and water mixing were made in autumn 1996, at four coastal sites close to Bahía Tortugas, on the central part of the Baja California Pacific coast, to evaluate the influence of hydrodynamics on the transport of abalone larvae (*Haliotis* spp.). Current measurements and full-scale Lagragean experiments on surface-water transport were carried out during the main spawning season of abalone in the area. Wind and wave-driven currents appear to be the most important factors affecting larval transport in this coastal area, and tidal currents do not appear to play a dominant role. Additional echo sounding and aerial surveys confirmed that the reef topography and kelp beds attenuate current velocity. The hypothesis of larval dispersion is that during a typical 3 to 5 day pelagic period, larval and postlarval stages might be retained primarily in areas close to parental reefs. Flushing time in Bahía Tortugas was evaluated as 5 semi-diurnal tidal periods. Sufficiently intensive currents at the mouth of the bay (up to 25 cm s^{-1}) may complicate larval interchange between the northern and southern vicinities of the bay.

SIZE-STRUCTURED MODELS OF ABALONE POPULATIONS WITH A CRYPTIC COMPONENT TO THE STOCK. **Malcolm Haddon,** University of Tasmania, TAFI/ Marine Research Laboratories, Nubeena Crescent, Taroona, TAS 7053, Australia.

Size-structured models are often used when attempting to model the population dynamics of commercial stocks of abalone. These models are based around applying a projection matrix (P) to a vector of the population size distribution for each time period of growth ($N_{t+1} = PN_t$). The projection matrix is generated by multiplying a growth transition matrix (G) by a survivorship matrix (S) and adding a recruitment matrix (R) thus: $N_{t+1} = (GS + R)N_t$ or $N_{t+1} = (GS)N_t + R$, depending on the timing of processes in the model. The survivorship matrix is a combination of natural mortality and fishing mortality as applied through the filter of a selectivity curve. The repeated application of such a projection matrix and recruitment enables the expected size distribution of the modelled stock to be followed through time. Such simplistic models attempt to model the total population but cannot accommodate the often significant proportion of the abalone population that is cryptic (non-emergent, perhaps under rubble or boulder fields). Assessing only the emergent population would tend to underestimate both survivorship and recruitment. Alternative models, which include this non-emergent sector of the population, are described, with their respective strengths and weaknesses. Unfortunately, the field observations needed to fit these alternative models suggest that successful modelling of both the cryptic and emergent components of each population will require more than size information alone.

GENETIC SUBDIVISION OF THE ABALONE *HALIOTIS ROEI* IN SOUTH WESTERN AUSTRALIA. **A. T. Hancock,** WA Marine Research Laboratories, PO Box 20, North Beach, Western Australia.

Population structure of the abalone *Haliotis roei* in south western Australia was investigated genetically using starch-gel electrophoresis. Eight polymorphic enzymes were examined in 624 animals from 10 populations. Samples covered 3000 km of coastline, from Shark Bay in Western Australia to West Island in South Australia. Replicate samples were collected from 2 sites at 12 month intervals. The average Fst was 0.009 with 5 of the 8 loci showing significant variation in allelic frequencies. Sites showed no striking geographic trends in allelic frequencies or apparent clustering of sites using multidimensional scaling of Gst as a measure of genetic dissimilarity. A population structure of isolation-by-distance was evident when pairwise measure of Gst between populations were plotted against geographic distance. This relationship was evident beneath relatively high levels of variability in some pairwise comparisons of Gst for sites separated by small distances. The area of complete genetic mixing, or neighbourhood size, was estimated from pairwise Gst calculated for replicate samples at the same site. This technique estimates the size of the genetic neighbourhood to be less than the distance between the two nearest sites, or 13 km. The apparent contradiction between relatively high levels of gene flow across the species distribution, as indicated by a low average Fst, and substantial heterogeneity between sites separated by 10's of kilometers, is discussed in the context of the species biology. Conclusions highlight the common conceptual difficulty presented by relatively high levels of gene flow maintained despite the apparent isolation of local populations, which are primarily dependent on local recruitment for their continuation.

THE EXPERIMENTAL CULTIVATION OF THE SOUTH AFRICAN KELP *MACROCYSTIS ANGUSTIFOLIA*. **M. N. Harper, D. W. Keats,** and **R. J. Anderson,** Botany department, University Western Cape, P.O. Box X17, 7535, South Africa.

Kelps are the basis of a number of commercial products, including alginate, plant growth substances, beauty products, and food additives. More recently, in South Africa, kelps have become highly sought after as feed for the abalone mariculture industry. The potential of low volume, high value products such as medical alginate, as well as the available local market for abalone feed suggests that the commercial cultivation of kelps may be economically viable in some areas. This study examines the experimental cultivation of the kelp *Macrocystis angustifolia*. The kelp is cultivated using various techniques such as tank, raft, mist, spray, and laboratory cultivation. At present, only laboratory cultivation of gametophyte stage to sporophyte stage has been completed, using nutrient enriched seawater as a growth medium. Growth was consistent and favourable. Kelp will be cultivated on rafts in St Helena Bay and harvested kelp will be tested for alginate quality following purification using (a) viscometry, (b) mannuronic acid:guluronic acid ratios and (c) protein determination. In addition, properties of kelp important in terms of food quality for abalone will be investigated. These experiments will be used to determine which cultivation method would offer the highest quality of kelp for either alginate production or abalone feed.

EFFECT OF OXYGEN SUPERSATURATION AND TEMPERATURE ON JUVENILE GREENLIP, *HALIOTIS LAEVIGATA*, AND BLACKLIP, *HALIOTIS RUBRA*, ABALONE. **J. O. Harris, C. M. Burke, S. J. Edwards,** and **D. R. Johns,** School of Aquaculture, University of Tasmania, P.O. Box 1214, Launceston, Tasmania, Australia.

The growth and survival of greenlip and blacklip abalone were investigated in terms of their responses to high levels of dissolved oxygen (98–123% saturation). Blacklip abalone, *Haliotis rubra*, held at 17 °C and 99% oxygen saturation, grew significantly faster than all other treatments of blacklip abalone held at 19 °C, and significantly faster than blacklip abalone maintained at 107% oxygen saturation and 17 °C. Both temperature and oxygen saturation significantly affected the survival for this species. Blacklip abalone held at 19 °C had significant mortalities for both 98% oxygen saturation and 123% oxygen saturation when compared with mortality at 106% oxygen saturation. Oxygen consumption rates were depressed in supersaturated conditions for both species. No significant differences were noted for greenlip abalone, *Haliotis laevigata*, within the range tested in terms of growth rates, food consumption rates or survival, indicating more tolerance for these conditions than blacklip abalone.

AN OVERVIEW OF STATE AND NON-STATE RESPONSES TO ABALONE POACHING IN SOUTH AFRICA. **M. Hauck,** Institute of Criminology, University of Cape Town, South Africa.

Abalone poaching has escalated in South Africa over the last ten years, contributing to controversial political debates and heightening concern over the future of the abalone fishery. With ongoing conflict between the informal fishers, the commercial sector and the government, the last five years has spurred a suite of diverse responses. State-intervention has focused on

methods of policing, ranging from reactive to proactive strategies. These have included the implementation of training in the Western Cape province as a means of building capacity and increasing the effectiveness of law enforcement partners. In addition, non-state initiatives have been developing to mobilise communities to take action and to investigate other alternatives for fisheries compliance. The organisation of Seawatch has developed with one community as a means of addressing poaching activity at a local level. Residents have decided to take responsibility for building relationships with the authorities, compiling information and exploring local mechanisms for managing marine resources in its area. Independent research has also investigated the socio-political circumstances involved in poaching activity and has explored alternative possibilities for resource management. These strategies, which focus on both deterrence and voluntary compliance, seem to encompass important dynamics highlighted by fisheries compliance theory. However, the effectiveness of these initiatives has not yet been suitably evaluated. The appropriate balance between this 'carrot and stick' approach is still being explored in South Africa and important loopholes have been identified in each. One questions whether the political climate is conducive, and the economic resources available, to find this balance before the imminent collapse of the fishery.

ONTOGENETIC TRENDS OF SHELL BIOMINERALIZATION IN ABALONE, *HALIOTIS DISCUS HANNAI* INO. G. He and **K. Mai,** Aquaculture research laboratory, Ocean University of Qingdao 266003, China.

In the present study, the abalone *Haliotis discus hannai*, with different shell lengths (8, 14, 25, and 55 mm) was used for mineralogy and minor element composition study by X-ray diffraction and ICP. Besides calcite and aragonite, dolomite (CaMg(CO3)2) was detected for the first time in abalone shells. No calcite was detected in the 8-mm shell. From 14 to 55 mm shells, the ratio of calcite increased steadily from 1.6% to 13.6%. Correspondingly, the ratio of aragonite decreased from 95.3% to 83.9%. Generally, from 8-mm to 55-mm shell length, the concentration level of Mg, Mn, and Fe increased, while Zn and Cu dropped. Al and Na increased slightly with shell growth. The possible reasons for these variations are discussed in this paper. A characteristic comparison of shell matrix proteins among the different sizes of abalone was also conducted to reveal the changes in shell biomineralization with abalone growth. The apparent molecular weights of matrix proteins increased with shell growth when determined by gel-filtration. When separated by SDS-PAGE, four bands were detected in the 14-mm sample, i.e. 7.5 kDa, 18.3 kDa, 28.5 kDa, and 30.9 kDa. In the 25-mm sample, a 34.9 kDa band was detected in addition to bands detected in the 14-mm sample. In the 55-mm sample, 23.3 kDa and 46.4 kDa bands were detected in addition to bands detected in the 25-mm sample. The expression amount of 28.5 kDa protein was found to increase dramatically with development. These results demonstrate a hierarchical change in shell biomineralization in abalone development.

GROWTH REDUCTIONS IN GREENLIP (*HALIOTIS LAEVIGATA*) AND BLACKLIP (*HALIOTIS RUBRA*) ABALONE RESULTING FROM CHRONIC EXPOSURE TO SUBLETHAL COMBINATIONS OF ELEVATED AMMONIA AND LOW DISSOLVED OXYGEN LEVELS. S. Hindrum, C. Burke, S. Edwards, and **D. Johns,** School of Aquaculture, Tasmanian Aquaculture Fisheries Institute, University of Tasmania, PO Box 1214, Launceston, 7250, Tasmania, Australia.

Six groups of cultured abalone juveniles (1 control = Treatment 1, 7.61 ppm dissolved oxygen (DO), 5.62 ppb free ammonia nitrogen (FAN)) of two species were held in a flow-through bioassay system for 6–8 weeks and exposed to various combinations of dissolved oxygen and FAN in a factorial design as follows: Treatments 2, 3, 4 = 6.0 ppm DO and 32, 50 and 196 ppb FAN respectively, Treatments 5, 6 = 4.3 ppm DO and 32 and 50 ppb FAN respectively (actual measured values). Sand-filtered oceanic water was used to supply all treatments, ammonia being added to Treatments 2–6 as ammonium chloride, mixed into individual reservoirs for each treatment. Each reservoir supplied three replicate tanks through a 1.8 m constant head column in which DO was adjusted using a mixture of oxygen and nitrogen. Each tank held 15–20 abalone of each species in two separate cages, which were suspended in the water column.

For both species, at both oxygen levels, growth in terms of either specific growth rate for length (SGR-L) or specific growth rate for weight (SGR-W) tended to decline as FAN increased, with a significant interaction between DO and FAN for both species ($P < 0.005$). For greenlip abalone, SGR-L was significantly higher for Treatment 1 than for Treatments 2–6 (0.12% d^{-1} vs. 0.106, 0.058, 0.043, 0.065, and 0.081% d^{-1} respectively). For SGR-W, Treatments 5 and 6 were significantly higher than Treatments 1–4, and Treatments 1 and 2 were significantly higher than Treatments 3 and 4 (0.44 and 0.43% d^{-1}, 0.32 and 0.28% d^{-1}, 0.09 and 0.16% d^{-1} respectively). For blacklips, Treatments 1 and 2 grew significantly faster in terms of SGR-L than Treatments 3–6 (0.034 and 0.036% d^{-1} vs. 0.024, 0.013, 0.022, 0.014% d^{-1} respectively), with Treatment 4 significantly lower than Treatments 3, 5 and 6. In terms of SGR-W, Treatments 3 and 4 were significantly lower than Treatments 1, 2, 5 and 6 (−0.025 and −0.051% d^{-1} vs. 0.16, 0.074, 0.19 and 0.11% d^{-1} respectively).

These results confirm the sensitivity of these species to chronic exposure of even modest reductions in water quality.

RESEARCH INTO A NEW TECHNOLOGY FOR ARTIFICIAL ABALONE BREEDING. Z. Hongen, Director of Dalian Fisheries Institute, Fujiazhuang 267, Xigang District, Dalian, China.

A hybridization technique is used to increase resistance against disease in cultured abalone, by cross-breeding of *Haliotis discus* Reeve broodstock from Japan with *Haliotis discus* Lannai from Dalian, China. Half of the total breeding area is applied to collect seeding. The other half of the area is used to culture benthic diatoms so that juvenile abalone transferred to the plates will have sufficient diatoms to feed.

The use of the hybridisation technique extended the time during which abalone feed on diatoms, and improved the growth of juveniles. Survival rates increased form 20% to 80%, and production of abalone from 1500 m^{-2} to 5000 m^{-2}, accompanied by the best growth rates. Average shell length also increased, from 1.5 cm to 2.0 cm. The growth rates increased by 33%.

ASSESSMENT OF THE EFFECTS OF FISHING INTENSITY ON STOCK LEVELS IN THE ABALONE DIVING FISHERY. T. Horii, National Research Institute of Fisheries Science, 6-31-1 Nagai, Yokosuka, Kanagawa, 238-0316, Japan.

Based on catch-effort data on the abalone diving fishery over a 20-year period (1978–1998) in Ojika Island off Nagasaki, the effects of fishing intensity in a particular year on the stock level in the subsequent year were examined. Owing to the extensive range of the annual total effort of 2156–3798 worker-days, it was practically difficult and unreliable to assess the stock level with mean annual values of catch per unit effort (CPUE). As such, a cumulative catch when the cumulative effort reached 2000 worker-days ($C_{2000,t}$) was defined as an alternative index of stock abundance in t year. In addition, the ratios of $C_{2000,t}$ to mean values of $C_{2000,t-1}$, $C_{2000,t-2}$ and $C_{2000,t-3}$ (last three years) were calculated as a relative index of stock abundance in t year (NIt). Furthermore, the ratios of total effort in t year to mean values of total effort in the last three years were calculated as a relative index of fishing intensity in t year (EIt). Here, At is denoted NIt/EIt; the lower the At value, the higher the tendency of over-exploitation. Relationships between $At-1$ and NIt showed a highly positive correlation (less than 1% level of significance); the stock level on any particular year was markedly affected by the fishing intensity of the preceding year. It is therefore necessary to adjust fishing intensity, depending on the stock level within the fishing period, to avoid over-exploitation in the subsequent year of limited abalone resources.

ANALYSIS OF THE SUITABILITY OF AUSTRALIAN FORMULATED DIETS FOR THE AQUACULTURE OF THE TROPICAL ABALONE, *HALIOTIS ASININA* LINNEUS. D. J. Jackson, K. Williams, and **B. Degnan,** Department of Zoology and Entomology, University of Queensland, Australia 4072.

The tropical abalone, *Haliotis asinina* Linnaeus, has recently been recognised for its potential as a new aquaculture species within Australia. However it is not known whether any of the commercially available diets formulated for temperate species and temperate conditions are suitable for this species. A growth assay testing four commercial Australian diets and a seaweed, *Gracilaria edulis*, was conducted to investigate the suitability of temperate formulated diets for *H. asinina*. The trial was run for 6 months at 28 °C following a nutritional acclimation period of 41 days. Abalone with a starting shell length of 18.3–2.76 mm and weight of 1.32–0.577 g were used. A formulated diet that performed well under the experimental conditions was identified by measuring growth (shell length and weight) and survival. The presence or absence of gonad tissue was monitored from the third month of the trial onwards, and the gonad index (GI) was measured externally at the end of the trial. Animals maintained on the four formulated diets matured precociously while only one female maintained on the *G. edulis* diet developed gonad tissue by the end of the trial. There were no significant differences in GI between formulated diets. However, histological examination of female gonads revealed significant structural differences that reflected diet quality (as measured by growth and survival). Animals maintained on poorer quality diets showed evidence of degenerated gonads with large populations of macrophages suggesting resorption of gonadal tissue. Animals maintained on higher quality diets had normal oocytes and gonad structure, and did not display this macrophage activity. The efficiency of dry matter food conversion by abalone fed each of the diets was measured during a two week period of the growth assay, revealing no significant differences. During this period, the nightly weight of food ingested per experimental unit was measured. A gradual increase in the weight of food ingested (expressed as a percentage of wet body weight) over the nights leading up to a full moon (coinciding with spawning patterns observed in adults) and a subsequent decrease was observed for animals fed the highest quality artificial diet.

SPAWNING INDUCTION OF *HALIOTIS AUSTRALIS* USING DIFFERENT CHEMICALS AND GANGLIONIC SUSPENSIONS. N. M. J. Kabir and **P. V. Mladenov,** Department of Marine Science, University of Otago, P.O. Box 56, Dunedin, New Zealand.

Injections of serotonin (5-hydroxytryptamine, 10^{-3}M), dopamine (3-hydroxytyramine 10^{-3}M), prostaglandin E_2 (10^{-5}M), de-ionised fresh water, filtered seawater, cerebral (CG) and pleural-pedal (PPG) ganglionic suspensions (10 ganglia per mL of water) were assayed as inducers of spawning in the yellowfoot abalone, *Haliotis australis*. Injections were made three times, once a day, into the haemocoel near the cerebral ganglia of males and females at a dose of 0.1 mL per day. A batch of 98 animals with fully ripe gonads (n = 6–10 per trial) was selected. Changes in the body weight and release of gametes were monitored regularly for each individual for four days. 100% of the females and 67% of the males injected with filtered seawater spawned, 50% of the females and 25% of males injected with serotonin spawned. Prostaglandin E_2 did not induce the release of gametes. 50% of the females treated with dopamine spawned a small number of eggs while the males did not respond. Males did not respond to injection of suspensions from any kind of ganglia and 20% of the females spawned a few eggs in response to CG from females and PPG from females. 40% of females spawned in response to PPG from males. Injection of de-ionised fresh water caused no weight changes whereas filtered seawater caused a reduction in weight. Prostaglandin treated animals gained weight on the 2nd day but lost weight over subsequent days. Only the females gained weight in the dopamine treated group, and both males and females gained weight in the serotonin treated group. In the case of ganglionic injection, males treated with male CG or PPG gained more weight than their female counterparts and the females treated with female CG or PPG gained more weight than males. The increase in mean body weight of animals was followed by a swelling and softening of the ovaries, possibly due to an increased water content in the ovaries. It seems likely that uptake of water in the ovary is a physiological precursor to spawning.

EFFECTS OF DELAYED METAMORPHOSIS ON SURVIVAL AND GROWTH OF NEWLY METAMORPHOSED *HALIOTIS DISCUS HANNAI*. T. Kawamura, H. Takami, and **Y. Yamashita,** Tohoku National Fisheries Research Institute, 3-27-5 Shinhama, Shiogama, Miyagi 985-0001, Japan.

Swimming larvae of *Haliotis* species delay metamorphosis if they fail to contact an appropriate environmental stimulus. The effects of delayed metamorphosis on survival and growth of post-larval *Haliotis discus hannai* were examined. Competent larvae were induced to metamorphose at 5, 10, 15, and 19 days after fertilization by the addition of 1 μM γ-aminobutyric acid (GABA). Larvae in another group were maintained until individuals metamorphosed spontaneously. Metamorphosed individuals (post-larvae) were reared in the laboratory and fed on a benthic diatom *Cylindrotheca closterium*. Starved post-larvae were reared in equivalent conditions, but without any food. All experiments were conducted at 20 °C. Metamorphosis, survival, and growth rates (determined from shell length) were measured.

The percentage of metamorphosed individuals 2 days after the addition of GABA increased with the length of larval swimming period; larvae that were 5 and 19 days old when induced to metamorphose showed 19 (±5.6; SE) and 96 (±3.6)% metamorphosis rates, respectively. The percentage of post-larvae that metamorphosed spontaneously increased after 17 days from fertilization and reached 96 (±3.0)% at 24 days post-fertilization.

Survival rates of fed post-larvae depended on the larval swimming period. For larvae that swam for ≤15 days, more than 80% of post-larvae survived the 20 day experimental period. In contrast, survival was only 57 (±2.9)% for 19 day old larvae. Post larval growth rates did not differ significantly for larval swimming periods ≤15 days. However, the growth rate of post-larvae from 19 day old larvae was significantly lower than that of 5 and 10 day old larvae. In the starved treatments, survival rate was lower, and the final shell lengths of the dead animals were less, as larval period became longer.

These results indicate that post-larval *H. discus hannai* are able to survive and grow normally within a larval period of 15 days (at 20 °C) if given adequate food. Nineteen days of larval swimming reduced post-larval survivorship and growth rate, suggesting the diminishment of larval yolk as an initial energy source for the metamorphosed post-larvae.

NON-DESTRUCTIVE DNA TYPING IN ABALONE HATCHERY MANAGEMENT APPLICATIONS. R. I. Lewis, E. G. Hall, and **J. S. Bee,** Division of Aquaculture, Dept. of Genetics, University of Stellenbosch, Private Bag XI, Stellenbosch 7602, South Africa. **N. A. Sweijd,** Dept. of Zoology, University of Cape Town, South Africa.

Genetic management of hatchery stocks is often given a low priority, since initially the most dramatic production gains can be achieved through improved husbandry practices. In the long term however, genetic management becomes highly desirable, not only to evaluate and exploit the productivity increases available from selection programmes, but to avoid productivity losses from inadvertent inbreeding and loss of diversity (and hence reduction in improvement potential) through genetic drift. Genetic management is considerably enhanced by the use of molecular tools that can estimate such characteristics as genetic variance, relatedness among individuals and populations, and levels of inbreeding. We

used PCR amplification of mtDNA to estimate levels of genetic diversity within and between two broodstock collections to evaluate their potential for short term heterotic exploitation. DNA was extracted from small tissue samples taken from mantle tentacles of sexually mature animals, which were later spawned—i.e. sampling was non-destructive facilitating future broodstock management based on known genetic background. Eggs, sperm, and subsequently larvae from pair matings were collected to investigate DNA extraction procedures, and to confirm the mode of mitochondrial DNA inheritance. F1 hatchery stocks were sampled to evaluate the genetic effects of current broodstock management practices.

GENETIC ANALYSIS OF A CULTURED POPULATION OF THE RED ABALONE, *HALIOTIS RUFESCENS*, IN MEXICO. **A. L. Licona Chávez** and **M. A. del Río Portilla**, Centro de Investigación Científica y de Educación Superior de Ensenada, Km 107 Carr. Tijuana-Ensenada, Ensenada, B.C. México, A.P. 2732, Ensenada, México, 22800.

The red abalone, *Haliotis rufescens*, is one of the species that has been cultivated and successfully commercialized in the USA and Mexico. However, there are not many known genetic studies on the cultured populations. Allozyme electrophoresis was used to characterize genetically the population of red abalone cultured by the "Abulones Cultivados, S. A." company. Five batches (range 1.6–9.5 cm) were sampled, with a total of 298 abalones, from the grow-out facilities in the Isla de Todos Santos, B. C. and another two batches were from the inland facilities in Ejido Eréndira, B. C. (range 0.49–1.42 cm) were also sampled, with a total of 298 abalones. As a result of farm procedures, these two batches (J and K) were divided into three (6, 8, and 10 mm) and two (6 and 8 mm) groups, and so were genetically analysed separately.

Thirteen allozyme loci were scored for all organisms; three were monomorphic and the others were polymorphic, although in some batches few loci were monomorphic. Only three loci did not adjust significantly to the Hardy-Weinberg mode, of which two showed heterozygote excess and one showed heterozygote deficiency. From a total of 74 cases, 25 showed heterozygote deficiencies, while 43 had heterozygote excess. Thus, in general, there was not heterozygote deficiency. Unbiased heterozygosity (H) was evaluated for each subpopulation and, on average, H was slightly larger than that of the red abalone cultured in California. H showed a tendency to increase with size, J6 heterozygosity was smaller than J8 and the latter was smaller than J10, and, also, K6 heterozygosity was smaller than K10. However, there was no significant positive correlation between individual heterozygosity and size in any case. In general, "Abulones Cultivados" population was considered to be in equilibrium and with high genetic variability.

REPRODUCTIVE PERFORMANCE INDICES BASED ON PHYSICAL CHARACTERISTICS OF THE FEMALE BLACKLIP ABALONE *HALIOTIS RUBRA* **L. M. Litaay** and **S. S. De Silva**, School of Ecology & Environment, Deakin University PO Box 423, Warrnambool, Victoria, Australia 3280.

Selection of abalone broodstock from the wild is often based on external appearances. The criteria used are size, colour and shape of the gonad. However, performances such as egg fertilisability, hatching rates and larval survival of selected broodstock vary widely. The present study, instigated to develop easily useable indices based on physical characteristics for assessing the potential reproductive performance of female abalone was conducted on wild-caught, artificially propagated blacklip abalone, obtained from coastal waters (142°15′E, 38°21′S), Australia. Shell characteristics, length (SL), width (SW), height (SH), and total weight (W) were determined. Females were spawned using a combination of desiccation and UV irradiated methods. The fertilised eggs from each spawning were incubated, hatched, and larvae reared separately in a flow-through system. The criteria used for assessing reproductive performance were: fecundity, fertilisability, hatchability, and pre-settlement survival. The results showed that shell characteristics can be used as a predictor of the reproductive performance. In addition, a combination of physical characters, such as SL and SW appeared to be good indicators of reproductive performance predictability. Accordingly, a number of highly non-linear regressions incorporating egg characteristics to reproductive performance were developed. A ratio between some of the shell characteristics can also be used as indices for broodstock assessment. We also found that fertilisability was positively correlated with hatchability and larval survival ($P < 0.01$).

IgE AND MONOCLONAL ANTIBODY BINDING TO ABALONE AND OTHER MOLLUSC ALLERGENS. **A. L. Lopata, B. Fenemore**, and **P. C. Potter**, Allergology Unit, Groote Schuur Hospital, Observatory 7925, South Africa.

World-wide, seafood represents one of the most important groups of allergens in the induction of food allergy. With the increased consumption of seafood, the rate of adverse reactions is believed to be rising. In recent years, patients with adverse reactions to abalone ('perlemoen', *Haliotis midae*) have been reported to the Allergology Unit with increasing frequency. Several immunoglobulin E (IgE) binding sites have been identified in extracts of abalone using SDS-gel electrophoresis and Western blotting. Two proteins with molecular weights of 38 kDa and 45 kDa were found to be the major allergens and appeared to very heat-stable. The later unique seafood allergen has been named Hal m 1 (according to the WHO/International Union of Immunological Societies). Hal m 1-like bands were also detected in other abalone and mollusc

species. In addition, allergens with different molecular weights that varied between the analysed subjects were demonstrated.

To be able to detect the same or similar abalone allergens specifically in other mollusc species, we generated monoclonal antibodies (MoAbs), using hybridoma technology. For this purpose, several abalone proteins were extracted after SDS-gel electrophoresis and used for the immunisation of mice. Monoclonal antibodies have the advantage that they are homogenous in immunoglobulin subclass specificity and bind to the same epitope.

Two cross-reacting proteins, with 42 kDa and 45 kDa, were recognised by MoAb 1.10 in most of the analysed mollusc species such as snail, limpet, and various mussel species. In contrast, MoAb 1.4 demonstrated binding only to the 42 kDa protein in abalone but not in other molluscs. These results demonstrated clearly that the two MoAbs, developed against the same purified protein from abalone, recognised different epitopes on the same protein. Furthermore, these MoAbs differentiate between closely related species, such as between the two South African abalone *H. midae* and *H. spadicea* and even between subspecies such as *H. discus hannai* and *H. discus discus*. This immunological technique can therefore be used for the differentiation of closely related mollusc species.

Further sequence analysis and epitope mapping of the allergens/antigens in abalone and other molluscs will advance our understanding of the molecular basis and pathogenesis of mollusc allergy.

IMMUNOLOGICAL DETECTION OF VARIOUS ABALONE SPECIES. **A. L. Lopata** and **T. Luijkx**, Allergology Unit, Groote Schuur Hospital, Observatory 7925, South Africa, **N. A. Sweijd** and **P. A. Cook**, University of Cape Town, Department of Zoology, Rondebosch 7701.

Proteins of any given organism are an expression of its genetic composition. Therefore, many of the methods employed in differentiating various animal species rely on different techniques of protein analysis. Electrophoretic separation of proteins according to their molecular weight was conducted using SDS-polyacrylamide gels. Species-specific banding patterns were observed. However, the differentiation of very closely related species did not always give satisfactory results. Novel immunological techniques were applied to allow clear identification between subspecies. Furthermore, the development of a field-based identification kit was anticipated.

Monoclonal antibodies were generated in mice against species-specific proteins of *Haliotis midae*. Over ten commercially used abalone species from South Africa, Australia and Japan were analysed by Western blotting. Specific antibody binding identified mainly proteins with about 38 kilodalton (kDa) and 45 kDa. Furthermore, species-specific binding patterns distinguished between very closely related subspecies, such as between *H. discus discus* and *H. discus hannai* as well as between *H. diversicolor diversicolor* and *H. diversicolor supertexta*. These immunological techniques present many advantages, among them their relative simplicity and accuracy in identifying different abalone species.

EFFECT OF FORMULATED DIETS, FRESH SEAWEED AND TEMPERATURE ON GROWTH RATES, GONAD DEVELOPMENT AND SHELL FORMATION OF THE EUROPEAN ABALONE *HALIOTIS TUBERCULATA* L. L. M. López and **P. Tyler**, School of Ocean and Earth Science, University of Southampton, Southampton Oceanography Centre, European Way, Southampton, SO14 3ZH, United Kingdom.

Formulated diets have been shown to improve the growth rates of juveniles and young adults of species of *Haliotis*. When juvenile *Haliotis tuberculata* were fed on formulated diets, and cultured at 18° and 22 °C, our research showed early development of the gonad and deformation of the shell. Three different diets were evaluated and the growth rates (shell length and body weight) of the juveniles were obtained for each. The first diet was formulated with fishmeal (FM) as the main protein source, the second was an abalone commercial (CO) diet containing casein as the main protein source and the third was a mix of fresh seaweed (*Palmaria palmata* and *Ulva lactuca*, SW) used as a control. Seven-month-old juveniles (4.16-5.97 mm 14.33-30.12 mg) reared in our laboratory, were fed over a period of seven months and cultured at 15°, 18°, and 22 °C temperature.

During the first three months, the daily growth rates (shell length and body weight) were similar between formulated diets (FM and CO). A maximum growth rate of 136 μm day^{-1} and 3,091 μg day^{-1} was attained when cultured at 22 °C. From the fourth month, the growth rates decreased to 41 μm day^{-1} and 229 μg day^{-1}. At the same time the gonad began to develop in all animals (FM and CO) cultured at 18° and 22 °C. Whilest growth rates decreased, the gonad development, was notably more rapid, showing that a great part of the metabolised energy was diverted to reproduction. In our study, gonad development started at 1.09 cm shell length, and at eleven months old. The first juveniles spawned at the end of the first year. On the other hand, abalone fed on all three diets and cultured at 15 °C plus those on the SW diet cultured at 18° and 22 °C did not show gonad development. Their growth rates were low and constant throughout the experimental period.

87% of the population presented shell deformation after four months feeding on CO diet. In this context, a specific nutrient deficiency may occur when there is an imbalance in the proportion of that nutrient in an otherwise adequate diet.

THE USE OF ULTRASOUND IN THE TREATMENT OF SABELLID INFESTATIONS IN SOUTH AFRICAN ABALONE. **N. C. Loubser** and **N. Dormehl**, I&J Abalone Culture Division, PO Box 522, Gansbaai, South Africa.

Gill tissue of *Haliotis midae* and different life stages of the sabellid worm were exposed to ultrasound to investigate any destructive effects of micro-cavitation. Live abalone infected with the sabellid worm were also exposed to varying periods of ultrasound treatment to investigate the long-term effect of ultrasound cavitation on the reproductive rate of the sabellid worm.

Ultrasound treatment for one minute was enough to destroy isolated sabellid adults in seawater media in a test tube. The feeding crown of the sabellid adult was destroyed after a thirty second treatment. Ultrasound treatment for one minute was not adequate to destroy islolated sabellid larvae, sabellid eggs or the abalone gill tissue.

Individually marked abalone, 60 in total, were treated either completely submerged during the ultrasound treatment or partially submerged with only the shell in the water column during the treatment. Replicates were exposed for time intervals varying from 1 minute to 10 minutes in one minute increments. The abalone showed severe stress behavior during all treatments, with two of the shells coming off during the shell-only treatment. One month after the treatment, no sabellid eggs or larvae were present in the abalone shells. There were still sabellid adults present, with the greatest proportion of the adults having their feeding crowns destroyed. A very small proportion of the adults near the shell edge retained their normal size and colour and still had active feeding crowns. After three months, the treated abalone had an average growth rate of 3.16 mm/mth, whereas the untreated control still had an average growth rate of 1.32 mm/mth which was similar to the historical growth rate of the batch, viz. 1.26 mm/mth.

We speculate that the ultrasound micro-cavitation destroys the sensitive feeding crown of the worm, either completely or partially, and is therefore interfering with the worm's ability to feed properly and reproduce. A second treatment may be necessary to destroy the newly recruited adults which matured from the larvae and eggs. This research is now being continued on a much larger scale.

GROWTH AND AGEING OF PINTO ABALONE, *HALIOTIS KAMTSCHATKANA* IN BARKLEY SOUND, BRITISH COLUMBIA. **B. G. Lucas, A. Campbell, B. Clapp,** and **G. S. Jamieson**, Fisheries and Oceans Canada, Pacific Biological Station, Nanaimo, BC, Canada V9R 5K6.

A long-term tag recapture study of *Haliotis kamtschatkana* was conducted at three islands in Barkley Sound on the West Coast of Vancouver Island, British Columbia between 1991 and 1998. A total of 5 627 abalone were tagged, and 742 were recaptured. Time at liberty ranged from one day to 5 years, with the percent of total recaptures being 12% for less than one year, 72% for one year, 14% for two years and 2% for more than 2 years. Preliminary analyses indicated some differences in growth rates between sites and for different one-year periods, but not between males and females.

Instantaneous natural mortality rates for pinto abalone were 0.129 at Hankin Island, 0.157 at Turret Island and 0.139 at Dempster Island. The spire ring technique was used to age abalone shells from Dempster Island and provided an average growth rate of 6.25 mm per year, which was similar to rates determined from tag recapture growth increments. Von Bertalanffy growth parameters derived from tag recapture growth increments were most similar to those derived from age data when each prismatic ring was counted as one year of growth per ring (simple brown rings were not counted).

Damage from boring parasites and erosion had destroyed an average of 60% of the shell spires, limiting the region of the horizontal section of the spire where rings could be counted. Although *H. kamtschatkana* appeared to deposit one prismatic ring per year in British Columbia, further research is needed to determine the significance of fine rings, brown rings, boring parasites and erosion on the number of apparent rings.

THE EFFECT OF STOCKING DENSITY, TEMPERATURE AND LIGHT ON THE EARLY LARVAL SURVIVAL OF THE ABALONE *HALIOTIS ASININA* LINN. **J. A. Madrones Ladja**, Aquaculture Department, Southeast Asian Fisheries Development Center, Tigbauan 5021 Iloilo, Philippines.

Newly hatched early veliger larvae of the abalone *Haliotis asinina* were stocked at densities of 1000, 3000 and 5000 larvae/l at low (20–25 °C) and high (ambient, 28–30 °C) water temperature levels, in transparent (light) and black cloth-covered (dark) glass containers. Larvae were reared in UV light-irradiated seawater until pre-settlement stage. Aeration was not provided during the 20-h incubation period. A 3 × 2 × 2 factorial design with three replicates per treatment was followed.

The three-way ANOVA showed a significant interaction among the factors tested. Analysis showed that at a stocking density of 1000 larvae/l, there were no significant differences between temperatures, and between light or dark conditions. However, at densities of 3000 or 5000, significantly high survival rates were obtained at lower temperatures ($P < 0.001$), but no difference was detected between the light and dark conditions. At high temperatures, better survival rates ($P < 0.05$) were obtained at a stocking density of 1000 than at higher densities, and at light than at dark condition. At low temperatures, no significant difference between

densities or between light and dark conditions was detected. Analysis of data from the light or dark condition showed that at any of these conditions, larval survival was always higher at a stocking density of 1000 than at other densities. The stocking densities of 3000 and 5000 larvae/l were not significantly different from each other.

Therefore, during incubation of hatch-out larvae of *H. asinina* to pre-settlement stage, the optimum stocking density at high temperatures is 1000/l, in a light-penetrable rearing container. But when reared at higher stocking densities of 3000 or 5000, higher survival rates can be obtained at temperatures of 20–25 °C, in either rearing conditions tested.

GUAIACOL, A POWERFUL MODULATOR OF MOLLUSC SHELL BIOMINERALIZATION. **K. Mai** and **G. He,** Aquaculture research laboratory, Ocean University of Qingdao Qingdao 266003, China.

As a modulator of eicosanoids metabolism and an inhibitor of calcium flux, guaiacol was examined for its influences on shell biomineralization in abalone, *Haliotis discus hannai*. Juvenile abalone (14 mm in shell length) were fed on artificial diet with guaiacol at 10 mg/kg diet for 100 days. The concentration of calcium in shells decreased from 38.7% to 32.1%-a level similar to that in adult shells (55 mm in shell length). The concentration of zinc decreased from 53.4% to 39.3%; but other elements showed no significant difference. Compared to the control, the fraction of calcite increased dramatically from 1.5% to 11.5% similar to that in adult shells (13.2%). Similarly, the fraction of aragonite decreased from 93.2% to 85.1%. Compared to the control, the acidic amino acid content in shell soluble matrix proteins decreased.

EVALUATION OF SEASONAL BIOENERGETICS OF *HALIOTIS FULGENS* AND *HALIOTIS TUBERCULATA*. **S. C. McBride,** University of California Sea Grant Extension Program. 2 Commercial St. Suite 4 Eureka, California 95501. **E. Rotem, D. Ben-Ezra,** and **M. Shipgel,** Israel Oceanographic and Limnological Research, National Center for Mariculture P.O. Box 1212, Eilat 88112, Israel.

Bioenergetics (food ingestion and absorption, respiration, ammonia excretion, mucus production and growth) of the temperate water abalone species, *Haliotis tuberculata*, and warm water species *Haliotis fulgens*, were studied under summer and winter conditions in the Gulf of Eilat. Three sizes (mean ± S.D) of *H. fulgens* and *H. tuberculata* (0.2 + 0.03 g; 0.5 ± 0.02 g; 1.9 ± 0.1 g) were cultured for one year (July 1995 to March 1996) where they were fed *ad libitum* with the seaweeds *Ulva lactuca* and *Gracilaria conferta*. Growth was highest for both *H. fulgens* and *H. tuberculata* during winter (3 to 7 mg DW d^{-1} and 3 to 6 mg DW d^{-1}, respectively). In summer, *H. fulgens* increased in dry weight at a rate of 2-mg d^{-1} in all size classes while *H. tuberculata* lost weight but showed a small amount of shell growth in the early summer. Respiration was greater during summer for both species and all size classes. Assimilation efficiency was highest for small and medium sized abalone of both species, compared to large abalone (*ca.* 75% vs 60%) but did not differ significantly ($P > 0.05$) between seasons. On an energy basis, respiration, ammonia excretion, and mucus production accounted for 30% ± 2; 37% ± 1; and 57% ± 1 of the total energy (joules d^{-1}) absorbed by small, medium and large abalone, respectively.

These results suggest that the prevailing conditions in the Red Sea are more suitable for the culture and growth of *H. fulgens* and *H. tuberculata*.

THE ROLE OF A *RICKETTSIA*-LIKE PROKARYOTE IN WITHERING SYNDROME IN CALIFORNIA RED ABALONE, *HALIOTIS RUFESCENS*. **J. D. Moore, T. T. Robbins,** and **C. S. Friedman,** Bodega Marine Laboratory, 2099 Westside Road, Bodega Bay CA.

Withering syndrome (WS) is a chronic, progressive disease responsible for mass mortalities in wild populations of black abalone *Haliotis cracherodii* in southern California since the 1980s. Although the red abalone, *Haliotis rufescens*, appears more refractory to WS, farmers nevertheless suffered severe losses of red abalone with WS clinical signs during the 1997–1998 El Niño event. Our studies investigated the roles of a gastrointestinal *Rickettsia*-like prokaryote (RLP) and elevated temperature in WS. In one experiment, 60 red abalone were obtained from a culture facility with cool (14 °C) ambient water that had not experienced WS, although animals were known to harbor low-level RLP infections. One half of these animals were maintained at 14.7 °C (control) and the other half were brought to 18.5 °C (elevated temperature) to attempt induction of WS. After 220 days, those held at the elevated temperature showed higher mortality, more severe signs of WS and more severe RLP infections than those held in cool water. Signs of WS were strongly correlated with RLP infection intensity among the elevated temperature animals. To investigate this relationship under typical farm conditions, 70 red abalone were sampled from five farms before and during the 1997–1998 El Niño, and severity of WS clinical signs and associated histopathology were strongly associated with RLP infection intensity. In a separate study, the lack of requirement for physical contact between abalone for RLP transmission was shown by successful transmission to RLP-free red abalone held in separate tanks downstream from infected abalone. One year after initiation of a second, ongoing transmission study conducted at 18 °C, RLP-free red abalone that were injected with RLP-infected gastrointestinal tissue experienced 80% mortality with nearly all showing signs of WS and severe RLP infections, while those injected with a 0.1 μm filtrate of infected tissue as well as saline-injected and uninjected

control animals remained healthy. Collectively, these studies support the hypothesis that the RLP is the etiologic agent of WS, with temperature influencing expression of the disease. Cool water may provide a refuge from the pathogenic effects of the RLP although it is also possible that stressors additional to elevated temperature may influence the occurrence of WS.

HEALTH MANAGEMENT AND DISEASE SURVEILLANCE IN ABALONE, *HALIOTIS MIDAE*, IN SOUTH AFRICA. **A. Mouton,** Onderstepoort Veterinary Institute, Private Bag X05, Onderstepoort, 0110, South Africa.

Abalone production units in South Africa have participated in a health management programme since March 1999. The aim of the programme is to identify potential threats to the health of the animals and to determine suitable ways of managing such threats so as to minimise the impact on production. This aim is achieved by a comprehensive system of sampling for each production unit. Samples typically include animals from the weaning and grow-out sections, as well as eggs, newly hatched larvae and post settlement larvae. Animals which are seen to be suffering from disease are also submitted for examination. Methods of examination include histopathology, bacterial culture and scanning and transmission electron microscopy. Regular consultation with production managers is an essential component of the programme. Disease surveillance for the entire cultured abalone population is facilitated by the large number of animals examined together with the wide distribution of sample sites. By August 1999, approximately 450 weaner and adult animals were being examined each month. This figure is likely to increase to over 800 towards the end of 1999. Interesting findings from the programme include the occurrence of renal coccidia in *Haliotis midae*, the presence of an unknown rickettsia-like organism in the digestive gland, and protozoan parasites affecting various sections of the gut. Problems and challenges experienced by the health management programme include lack of information on abalone diseases in general and *H. midae* in particular, reliable sample transport over long distances, and standardisation of processing techniques for eggs, newly hatched larvae and post settlement larvae.

A COMPARATIVE SCANNING ELECTRON AND LIGHT MICROSCOPY STUDY OF THE EARLY LIFE STAGES OF THE SOUTH AFRICAN ABALONE, *HALIOTIS MIDAE*. **A. Mouton** and **J. F. Putterill,** Onderstepoort Veterinary Institute, Private Bag X05, Onderstepoort, 0110, South Africa.

The early life stages of abalone are often neglected when it comes to diagnosis of disease. Although mortalities in the hatchery and nursery account for 90% or more of the total mortalities during the production cycle, the reasons for these losses are not completely understood. A comparative study of scanning electron and light microscopy of the early life stages of the South African abalone, *Haliotis midae*, was undertaken to determine normal characteristics for the species. Animals processed for scanning electron microscopy (SEM) were fixed in 4% gluteraldehyde in 0.2 M sodium cacodylate buffer (pH 7.2 to 7.4). Samples were rinsed twice in sodium cacodylate buffer, after which they were routinely dehydrated through an ascending series of ethanols (50, 70, 90, 96, 100, 100, and 100%, 30 minutes per step). The samples were then critical point dried from 100% ethanol through liquid carbon dioxide in a Polaron Critical Point Drier (Watford, England). Dried samples were mounted onto SEM viewing stubs and sputter coated with gold. The samples were viewed at 3 to 8 kV acceleration voltage in a Hitachi S-2500 Scanning Electron Microscope. Animals processed for light microscopy were fixed in either 4% gluteraldehyde in 0.2 M sodium cacodylate buffer (pH 7.2 to 7.4) or Davidson's fixative. Samples were rinsed twice in distilled water to remove adhering detritus. A modified double embedding technique (Feist & Bucke 1983 as described in Austin 1989, *Methods for the Microbiological Examination of Fish and Shellfish*) was used prior to processing routinely for light microscopy. Sections were cut at 6 μm and stained with haematoxylin and eosin. Due to inaccessibility of SEM, light microscopy is commonly used. Advantages and disadvantages of both methods are discussed.

EXPERIMENTAL CULTIVATION OF THE KELP *ECKLONIA MAXIMA*. **D. C. Z. Norman, D. W. Keats,** and **R. J. Anderson,** Botany Department, University of the Western Cape, P.O. Box X17, Bellville, 7535.

Kelps form the basis of a number of commercial products, including alginate, plant growth substances, beauty products, and food additives. Unprocessed kelps are also used as feed in the abalone mariculture industry. Alginate has potential for the development of biomedical implants, but the product must be of a very high purity and reproducible quality. Therefore, it may be desirable to grow kelp under mariculture conditions to improve alginate quality and reproducibility of medical implants. Laboratory, tank, spray and raft cultivation techniques were investigated. The laboratory cultivation of gametophytes and sporophytes was successful, using enriched seawater medium, and the young sporophytes have been grown in spray and mist cultivation prior to out-planting on a raft in the sea. Characteristics of the mist and spray system that affect growth are currently being investigated. The growth of sporophytes on rafts in St. Helena Bay is reported here.

BIOLOGICAL REFERENCE POINTS FOR THE GREENLIP ABALONE (*HALIOTIS LAEVIGATA*) IN DIFFERENT HABITATS ACROSS ITS GEOGRAPHIC RANGE. **J. L. O'Loughlin**[1,2] and **S. A. Shepherd,** [1]South Australian Research and Development Institute, GPO Box 120, Henley Beach, Australia, 5022, [2]Australian Maritime College, PO Box 21, Beaconsfield, Australia, 7270.

The greenlip abalone (*Haliotis laevigata*) is an exploited sedentary benthic gastropod endemic to southern Australia, with a geographic range from eastern Bass Strait to Cape Naturaliste in Western Australia. A substantial number of greenlip abalone populations have been declining over the last 25 years despite management techniques such as minimum legal sizes and total allowable catches which control fishing mortality.

Using aging techniques to estimate growth and mortality, egg production levels in metapopulations of *Haliotis laevigata* were estimated from western, central and eastern populations in the species' range. Comparisons were made between egg production in stable and declining populations, with a view to estimating threshold levels of egg production necessary for sustainable exploitation. Results confirm previous suggestions that small metapopulations are more vulnerable than large ones. Threshold egg production levels of small metapopulations appear to be around levels of 60% whereas thresholds for larger metapopulations are about 45%. Populations occurring in bays or around islands that constitute larval traps are more resilient to fishing than those on low relief, rocky bottoms, distant form shore. Our results suggest that up to 20% higher levels of egg production need to be conserved in the latter kind of habitat.

DISTANCE-BASED ABUNDANCE ESTIMATION FOR ABALONE. **R. A. Officer,** Tasmanian Aquaculture and Fisheries Institute, University of Tasmania, Nubeena Crescent, Taroona, Tasmania 7053, Australia.

Indices of abundance are an important component of stock assessment models. Unfortunately, conventional attempts to estimate the abundance of abalone are hampered by the patchy spatial distribution characteristic of abalone. Fine scale mapping of abalone positions was used to evaluate alternative distance-based abundance estimators for abalone that better accommodate the aggregated distribution of abalone. Simulated quadrat sampling of a real abalone population was used to examine the accuracy, bias and sensitivity of the distance-based approach. Within each quadrat, the distances from a randomly selected point and abalone to the nearest abalone were used as the basis for an overall estimate of abundance. An iterative randomisation procedure was used to obtain confidence limits about abundance estimates. This ability to re-sample the observed population reduces the need for the extensive field sampling normally required to narrow the confidence limits of abundance estimates. The distance-based method was found to under-estimate consistently the true abundance of the population and therefore may not be useful as an indicator of absolute abundance. However, this bias does not diminish the potential of the method as a relative abundance indicator because the method is sensitive to changes in abundance. Sensitivity to changes in abundance was examined by simulating the effects of fishing at varying rates of exploitation above specified size limits. Simulated reductions at moderate levels of exploitation (10–20% overall reduction) showed that the method was able to detect changes in abundance. The ability of the distance-based method to detect fishing-induced changes in abundance that could occur in reality is discussed.

STATUS OF THE ABALONE FISHERY BETWEEN 1996 AND 1999 ON ABALONE LOCATIONS OF THE FISHING CO-OPERATIVE PRODUCTION SOCIETY "LA PURÍSIMA", S. C. DE R. L., BAJA CALIFORNIA SUR, MEXICO. **M. Ortiz Quintanilla, G. Lucero M.,** and **J. E. Patrón V.,** S. C. P. P. "La Purísima", S. C. de R. L. Calle Segunda y Soto No. 2048, Col. Obrera, CP.22830, Ensenada, Baja California, México.

This work describes the status of the abalone fishery (*Haliotis* spp) during the commercial seasons between 1996 and 1999 on abalone diving areas held by the "La Purísima", S. C. de R. L., fishing co-operative production society. From the season of 1996, total quotas officially suggested by National Fishing Institute (I.N.P-CRIP), and based on the results of an annual survey, were subject to additional modifications imposed internally by the co-operative itself, as a measure towards improving wild abalone stocks, through a more rigorous resource management process.

These measures included the aquaculture production and release of competent green abalone *H. fulgens* veliger larvae into the wild, as well as leaving a portion of the recommended commercial stock as part of the fishery reserve, and the designation of protected areas. The 1996 abalone catch, with a quota of 3 861 kg (shelled abalone weight) was 814 kg; as a measure of effort, this catch derived from 74 fishing trips, with catch per unit effort (CPUE) data, measured as kg/diving team/day, of 11 kg per 12 abalone divers per day.

Up until 1996, two abalone species were included in the fishery, namely *H. corrugata* Wood and *H. fulgens* Philippi. Between 1997 and 1999, catches of *H. fulgens* were small. However, between 1997 and 1999, catches gradually increased from 2 029 kg in 1997 to 3 097 kg in 1999, and with changes in fishing efforts from 74 to 102 trips in 1999, and the 1997 average of 26.7 kg/diving team/day increasing to 33.22 kg/diving team/day in 1999, based on four abalone fishing units. CPUE based on the average mass of abalone captures per minute was also compared.

ABALONE (*HALIOTIS MIDAE*) FARMING AND PARALYTIC SHELLFISH POISONING ON THE COAST OF SOUTH AFRICA. **G. Pitcher,[1] J. Franco,[2] K. Whyte,[3] and C. Viljoen,[4]** [1]Marine and Coastal Management, Private Bag X2, Rogge Bay, 8012, Cape Town, South Africa, [2]Instituto de Investigaciones Marinas, Eduardo Cabello, 6, 36208 Vigo, Spain, [3]West Coast Abalone, P.O. Box 185, Stompneusbaai, 7382, South Africa, [4]Jacobsbaai Sea Products, P.O. Box 837, Vredenberg, 7380, South Africa.

The abalone *Haliotis midae* forms one of the oldest fisheries on the South African coast, with present-day operations including recreational, subsistence and commercial activities. During the 1990s, land-based farming of this species also developed and has recently attained commercial scale production. In April 1999, routine monitoring provided evidence of the presence of PSP toxins in cultured abalone. Subsequent analysis of wild abalone collected from the West Coast also revealed the unexpected accumulation of PSP toxins in these non-filter feeding shellfish. Toxicity, as measured by the mouse bioassay, showed considerable variation between individual animals, with maximum values exceeding 1000 μg STXeq 100 g^{-1}. The observation of PSP toxins in abalone coincided spatially and temporally with blooms of the dinoflagellate *Alexandrium catenella*. Toxicity as measured by High Performance Liquid Chromatography was notably higher than that measured by the mouse bioassay. The toxin composition of the abalone was dominated by saxitoxin and therefore differed significantly from the toxin profile of *A. catenella*, indicating either a high capacity for transformation of PSP toxins by abalone or that *A. catenella* was not the source of the toxin. Investigation of the anatomical distribution of toxins revealed that they were not evenly distributed throughout the abalone tissues. The muscular foot, which contributes substantially to the total weight of the soft tissues and is the organ marketed for human consumption, makes a disproportionately low contribution to the total toxin content of the mollusc. To date, the inability of abalone to detoxify accumulated PSP toxins below the regulatory level threatens the future of the established abalone fishery and the newly developed aquaculture operations on the West Coast.

CLUES AND QUESTIONS FROM POPULATION MODELS APPLIED TO THE SOUTH AFRICAN ABALONE (*HALIOTIS MIDAE*) FISHERY. **É. E. Plagányi** and **D. S. Butterworth,** Marine Resource Assessment and Management Centre, Department of Maths & Applied Maths, University of Cape Town, Private Bag 7701, Rondebosch, South Africa.

South Africa's commercially important abalone fishery depends on a single species *Haliotis midae* and is managed by allocating total allowable catches (TACs), with a minimum size limit, in each of seven specific fishing zones (zones A-G). Uncertainty and concern has been expressed regarding the status of the resource because several of the zones are subject to particularly high levels of illegal fishing. The magnitude of the catch taken by the illegal sector is difficult to estimate because of the evasive behaviour of the illegal fishing community. A population modelling approach has been used to estimate the magnitude of the illegal catch as well as to assess the status and productivity characteristics of the resource. The model applied is a deterministic age-structured production model. By projecting abundance trends under alternative future catch levels, the model is currently used as a basis for developing management advice in four (zones A-D) of the seven fishery zones. Attention has focused on zone C in particular and model results suggest that the annual poaching catch has exceeded the commercial catch in recent years. Moreover, results suggest that continued depletion of the resource in this and other zones is likely unless takes by all sectors of the fishery are reduced in future. This paper summarises current progress in the application of an age-structured production model basis for managing the abalone fishery, discusses problems associated with the use of both CPUE and fishery independent indices in the modelling process, and offers suggestions for simulating a postulated recruitment decrease in zones C and D.

STRESS AND WEIGHT LOSS ASSOCIATED WITH HANDLING IN THE BLACKFOOT ABALONE, *HALIOTIS IRIS*. **N. L. C. Ragg, H. H. Taylor,** and **J. Behrens,** Department of Zoology, University of Canterbury, Private Bag 4800, Christchurch, New Zealand.

During the course of physiological investigations carried out at the University of Canterbury, it became apparent that the simple act of handling an abalone markedly affected the animal's physiological state. Thus we routinely include handling as a control treatment in experiments; the responses of these control animals form the basis of this presentation. Adult *Haliotis iris*, subjected to standardised handling involving gentle detachment from the tank bottom followed by 2 minutes inversion on blotting paper to drain branchial water before weighing, consistently lost wet weight. The weight of animals handled repeatedly in this way, at intervals of 0.5 hours or less, continued to fall over 3 hours to a plateau, 7–17% below their undisturbed weight. The rate of weight loss varied directly with handling frequency. When left undisturbed, initial weight was recovered over several days. Thus, the operational body weight and volume of an abalone is a function of the frequency of weighing and of the intensity of the associated disturbance. Volume reduction of a number of fluid pools could potentially contribute to the weight loss, e.g. extra-corporeal water between the mantle and shell, fluid within the lumen of the kidneys, crop and other regions of the gut, mucus release, cellular dehy-

dration, or extracellular fluid (blood) loss. More than half of the volume of an undisturbed *H. iris* is blood (inulin space = 52.2 ± S.E 3.0 ml/100 g wet tissue, or about 31% of total weight, including shell). Surprisingly, repeated handling caused a 27.5 ± 5.7% increase in the concentration of the abalone's oxygen carrying pigment, haemocyanin. A tiny fraction of this increase (about 0.3% elevation of total haemocyanin) could be attributed to release of concentrated haemocyanin stored in the vasculature of the left kidney. The major increase in haemocyanin concentration was quantitatively consistent with a decrease in blood volume equivalent to the weight lost. We conclude that the principal effect is a handling-induced ultrafiltration of the blood, perhaps via the kidneys. Attention is therefore drawn to the fact that, in addition to the stress associated with air exposure (desiccation, hypoxia, thermal shock) and manipulation (possible injury), handled abalone also endure a substantial alteration in blood composition and volume, which must profoundly perturb circulatory function, oxygen uptake and transport to the tissues. The commercial implications of reduced meat weight and compromised growth and survival will be discussed, as well as techniques to avoid direct handling of abalone.

TOXICITY AND TOLERANCE LEVELS OF AMMONIA IN ABALONE (*HALIOTIS MIDAE*). K. Reddy-Lopata,[1] A. L. Lopata, and **P. A. Cook,[1]** [1]University of Cape Town, Department of Zoology, Rodenbosch 7701, South Africa, [2]Allergology Unit, Groote Schuur Hospital, Observatory 7925.

Ammonia is the major end-product of protein metabolism. Therefore, it is important to determine the accurate levels of toxicity and tolerance of ammonia in abalone (*Haliotis midae*) to help improve the water quality in South African abalone farms. Specifically, this study looks at ammonia tolerance and toxicity levels of abalone in relation to body size, pH and temperature. Growth experiments show that high concentrations of ammonia retard growth.

Toxicity tests revealed that when abalone are exposed to sublethal levels of ammonia, the LC50 value (ammonia concentration that kills 50% of experimental animals) increases. A possible mechanism for this increased tolerance of ammonia involves the so-called stress or heat shock proteins (hsp's). Hsp's are activated not only by heat but by other physiological stress (such as the toxicant ammonium chloride used in these experiments). Hsp's are designated according to their molecular weights. The 70 kilodalton hsp has been reported in a number of invertebrates and a few molluscs (limpets and mussels) but not in abalone.

The presence of hsp's were determined using biochemical and immunological techniques such as gel electrophoresis and immuno blotting. In addition, species-specific low molecular weight proteins in abalone were also investigated. Hsp's are of importance as they may be indicators of chemical stress and could be utilised to control water quality in abalone farms.

STARVATION TOLERANCE OF POST-LARVAL ABALONE (*HALIOTIS IRIS*). R. Roberts and **C. Lapworth,** Cawthron Institute, Private Bag 2, Nelson, New Zealand.

Competent larvae (16 days old) were induced to metamorphose with 2 μM GABA. Post-larvae were either fed diatoms (*Nitzschia longissima*) or starved. In Experiment 1, post-larvae were starved immediately after settlement, for periods of 1, 2, 4, 8, 15, 20, 25, and 30 days. Starved post-larvae grew relatively well for several days after metamorphosis (averages of 10.4 and 17.8 μm shell length per day after 8 days, for two batches). Subsequent growth was minimal, averaging 1.7–0.7 μm/day over 6–7 days. There was no clear relationship between period of starvation and growth rate. Mean daily growth rate over 20–25 days after return to food ranged from ~15–22 μm/day.

The duration of starvation had a significant effect on survival. Survival of post-larvae fed after just 1–2 days of starvation was 90–100% after 3 weeks of feeding. Longer starvation periods gave progressively lower survival rates. Survival probabilities were standardised to a 22 day period, and the relationship between survival (in our experimental conditions) and starvation period was estimated by linear regression (r-square = 0.861, $P < 0.001$): Probability of survival to 22 days post settlement = 0.943 – 0.029x, where x is the period of starvation in days.

In Experiment 2, post larvae were fed for 3 weeks after settlement, then starved for 0, 3, 7, 14, or 21 days. Growth rates of starved post-larvae dropped dramatically, averaging only 5–6 μm/day in the first week (versus 30 μm/day in controls), and later declining to zero. Growth resumed within a week following return to food, but the 14- and 21-day starvation treatments took two weeks to reach growth rates comparable to controls. Survival rates after return to food were strongly influenced by the period of starvation. The no-starvation controls and the 3- and 7-day starvation treatments all had > 70% survival over 5 weeks after return to food. Survival in the 14- and 21-day starvation treatments was 15–20%, with almost all mortalities occurring in the first week after return to food.

A REVIEW OF LARVAL SETTLEMENT CUES FOR ABALONE (*HALIOTIS* SPP.). R. Roberts, Cawthorn Institute, Private Bag 2, Nelson, New Zealand.

Settlement of abalone larvae involves larval attachment (a reversible behaviour) followed by metamorphosis (which involves irreversible physical changes). Coralline algae induce settlement in

all abalone species tested. The speed/strength of the settlement response differs among abalone species, and some abalone species prefer certain coralline species. The settlement-inducing chemicals from corallines have not been identified. In one case, a GABA-mimetic peptide is implicated, while in another, halomethanes are thought to be critical. Corallines are generally regarded as unsuitable for use in hatcheries, but their potential use has not been fully evaluated.

Many abalone hatcheries rely on biofilms to induce larval settlement. The activity of biofilms appears to increase with their age. Ungrazed films are generally dominated by fast-growing benthic diatoms, and settlement on these films is variable and often low. Few diatom strains are consistently good for settlement, and strains that are excessively mobile, or form 3-dimensional colonies, can prevent successful settlement. The chemistry of settlement induction by biofilms, and the role played by bacteria, are poorly understood. Bacteria grown in pure culture have limited settlement-inducing activity.

Pregrazing by juvenile conspecifics improves the settlement-inducing activity of a biofilm. The mucus trails from the foot of grazers may contain chemicals (not identified) that trigger settlement. Alternatively, the mucus or the grazing activity may enhance the biofilm in ways that favour settlement.

Various pure chemicals induce attachment and/or metamorphosis of abalone larvae. They may bind to larval receptors (e.g., GABA) or act "downstream" of the receptors (e.g., compounds that depolarise membranes or alter levels of cyclic AMP or calcium). None of these chemicals is considered to be a natural settlement cue, and only GABA is used in abalone hatcheries.

The timing and end point of the abalone settlement response varies in cues, and among abalone species. Cues can combine synergistically to enhance settlement (e.g., GABA + diatoms, dibromomethane + mucus). Cues for attachment are more common than cues for metamorphosis.

CRYOPRESERVATION OF ABALONE (*HALIOTIS IRIS*) SPERM. **R. Roberts,[1] S. Adams,[2] J. Smith,[3] A. Pugh,[3] A. Janke,[1] S. Buchanan,[1] P. Hessian,[4]** and **P. Mladenov,[2]** [1]Cawthorn Institute, Private Bag 2, Nelson, New Zealand, [2]Dept. Of Marine Science, University of Otago, P.O. Box 56, Dunedin, New Zealand, [3]AgResearch, Private Bag 3123, Hamilton, New Zealand, [4]Dept. of Physiology, University of Otago.

We are attempting to develop commercially applicable protocols for cyropreserving gametes and embryos of abalone, mussels and oysters. This paper reports preliminary findings from abalone sperm cryopreservation. We induced ripe abalone to spawn, using the hydrogen peroxide methods. Spawning males were removed from water to "dry spawn", yielding undiluted sperm (1–1.7 × 10^{10}/ml). Small-scale (3 to 10 ml) fertilisation assays were used to cope with the large number of samples required. These small assays can produce lower fertilisation rates than large-scale fertilisations.

There are two published protocols for the cryopreservation of abalone sperm. Neither produced satisfactory results with *Haliotis iris* sperm, so systematic investigation of freezing methods was initiated. Various cryoprotectants, equilibration periods, diluents, cooling rates and sperm densities were tested. In the most successful treatments, sperm were viable and highly motile after cryopreservation, but their fertility was reduced. The highest fertilisation rates obtained with cryopreserved sperm were 20–40%. These percentages were only achieved at very high sperm concentrations (10^7/ml). Corresponding fresh sperm controls gave 70% fertilisation at 10^6 sperm per ml. The most successful treatments were those with high sperm densities, DMSO as cryoprotectant, and relatively slow cooling.

Flow cytometric analysis of sperm stained with SYBR-14 and propidium iodide was used to examine sperm membrane integrity. Many sperm were membrane-intact after exposure to cryoprotectants, and after some cryopreservation treatments. However, "Hobson Sperm Tracker" analysis showed that many cryopreserved sperm were immotile, and that the motile sperm rapidly lost their motility after dilution. The swimming velocity and linearity were lower for cryopreserved sperm than for fresh sperm. We are investigating possible causes of low fertility of cryopreserved sperm, including damage to the sperm's mitochondria, flagellum or acrosome.

SELECTING AND EVALUATING MARINE PROTECTED AREAS FOR ABALONE IN CALIFORNIA. **L. Rogers-Bennett,[1] P. Haaker,[2]** and **K. Karpov,[3]** California Department of Fish and Game, [1]Bodega Marine Laboratory, PO Box 247, Bodega Bay, CA 94923, [2]330 Golden Shore, Long Beach, CA 90802, [3]19160 S Harbor Dr., Fort Bragg, CA 95437.

Abalone populations have declined dramatically in California, resulting in the closure of the commercial and recreational fisheries south of San Francisco. Marine Protected Areas (MPAs) have been proposed as a tool to help restore declining abalone populations, *Haliotis* spp. but more information is needed to locate MPAs and to determine the efficacy of such areas. One simple yet practical plan for siting MPAs is to analyze historical cumulative catch

data to identify areas which once supported large populations of target species. To do this, we examined spatially explicit catch data from the commercial fishery (1950–1996) to direct the selection of MPAs for abalone in California. San Clemente Island was the area of peak abundance of the now endangered white abalone, *Haliotis sorenseni* and the soon to be listed black, *Haliotis cracherodi*, pink, *Haliotis corrugata*, and green, *Haliotis fulgens* abalone, making this island uniquely suitable as an abalone restoration MPA. We also examined fishery independent data which included abundances and size frequency distributions of abalone inside and outside MPAs to examine the efficacy of existing MPAs. We found that the Anacapa Island MPA in the Channel Islands, where abalone fishing is excluded, supports higher populations of abalone than fished sites. Furthermore, remote parts of MPA that are not under the observation of the reserve manager, failed to protect pink abalone stocks which declined to zero as did neighboring fished sites. Therefore, we caution that while abalone abundances may be higher inside MPAs, effective enforcement of these areas is critical to their success.

MORPHOFUNCTIONAL STUDY OF THE HEMOCYTES OF *HALIOTIS ASININA*: A PRELIMINARY REPORT. S. Sahaphong,[1] V. Linthong,[2] S. Apisawetakan,[2] C. Wanichanon,[2] S. Riengrojpitak,[1] V. Viyanant,[3] S. E. Upatham,[3,4] N. Kangwanrangsan,[1] T. Pumthong,[5] and P. Sobhon,[2] Departments of [1]Pathobiology, [2]Anatomy, and [3]Biology, Faculty of Science, Mahidol University, Bangkok, Thailand 10400, [4]Department of Biology, Faculty of Science, Burapha University, Chonburi, Thailand, [5]Coastal Aquaculture Development Center, Department of Fishery, Ministry of Agriculture and Cooperatives, Prachuapkhirikhun, Thailand 77000.

The hemocytes of the abalone *Haliotis asinina* were studied using light and electron microscopy in order to describe their main morphological features and to relate these to their roles in immune defense. The cells comprise two differentiated types: agranulocyte or hyalinocyte and granulocyte. The hyalinocyte shows the presence of several filopodia, a large nucleus with dense chromatin, a moderate amount of cytoplasm, microfilaments, oval and round shaped mitochondria with a rather dense matrix, a considerable amount of rough endoplasmic reticulum, a few cytoplasmic granules, coated pits and vesicles, phagocytic vacuoles and numerous large and small vacuoles. Like the hyalinocyte, the granulocyte processes similar cytoplasmic organelles but in fewer numbers, and has a peripheral organelle-free zone containing numerous dense granules of various types. The shape of the granules vary from round, oval to elongated forms. Several dense granules exhibit crystalloid substructures that show a close relationship to the plasma membrane.

MEAT QUALITY CHARACTERISTICS OF SOUTH AFRICAN ABALONE (*HALIOTIS MIDAE*). J. Sales, P. J. Britz, and **T. Shipton,** Department of Ichthyology and Fisheries Science, Rhodes University, P O Box 94, Grahamstown, 6140, South Africa.

Some meat quality characteristics related to post-mortem glycolysis, drip loss, cooking loss and objective tenderness were evaluated in adult wild abalone (*Haliotis midae*) from the Eastern Cape coast of South Africa. The onset of pH decline was found to be 17 h after shucking when abalone were kept at 7 °C, while the corresponding figure was 13 h at 16 °C. From the time of onset of pH decline till 63 h post-mortem pH decline could be modelled by the formula $pH = B_0 - B_1(1 - \exp(B_2 t))$, where B_0 is the estimated pH at time (t) = 0, $B_0 - B_1$ is the asymptotic minimum pH, and B_2 is a measure of the rate of pH decline. Asymptotic minimum pH were 5.73 ± 0.056 (n = 6) at 7 °C and 5.54 ± 0.105 (n = 6) at 16 °C, while the rates of pH decline were −0.149 ± 0.121 and −0.090 ± 0.037 respectively. Drip loss (DL) over time could be described by the exponential model $DL = a + b(1 - \exp(-ct))$, where a is the intercept at time (t) = 0 (set to 0 in the present study), a + b is the asymptotic maximum drip loss and c is a measurement of the rate of drip loss. No differences ($P > 0.05$) could be found in either b (7.11 ± 3.001 vs 6.52 ± 1.278) or c (0.031 ± 0.031 vs 0.088 ± 0.075) between storage at 7 or 16 °C respectively. Abalone frozen at −20 °C immediately after shucking had a higher pH_{7days} ($P < 0.05$), but a lower Instron value (more tender) than those kept at 7 and 16 °C respectively ($P < 0.05$), indicating that toughness associated with rigor in red meat is absent in abalone meat. However, this has to be investigated regarding crumbling in fast frozen abalone meat. The present study presented the basic post-mortem pH decline and drip loss incline of South African abalone (*H. midae*) meat that could be used as a baseline for successive studies on manipulation of these parameters.

POST-SETTLEMENT OBSERVATIONS OF EZO ABALONE, *HALIOTIS DISCUS HANNAI* IN CONJUNCTION WITH FLORA. R. Sasaki, Miyagi-Prefectural Sea-Farming Center, Maeda, Yagawa-hama, Oshika-chou Miyagi-ken, 986-2402, Japan.

Natural rates of spat settlement of Japanese abalone (*Haliotis discus hannai*) on crustose coralline boulders were measured at various sites in the bay after larval monitoring. Averaged density was recorded as 220 inds m^{-2}, with 510 ± 20 μm shell-length along the sea-bottom from 150 m (depth 5 m) to 200 m (depth 7 m) distance from the shore. Daily mortality and growth rates were respectively calculated as 13% and 38 μm within a month after settlement. In some cases, dead shells of ca.500 μm length were found in the samples from crustose coralline boulders. These were

regarded as the first critical size caused by a starvation. Small *H. discus hannai* juveniles were primarily distributed around the depth layer of 5–6 m at the outer site and 1–2 m at the inner site of the bay, which coincides with the lowest distribution of *Eisenia*. The appearance of 1 year abalone was in the following proportions: 6% in *Eisenia* algal forest, 49% in the boundary zone and 25% in the crustose coralline algal area. In conjunction with the flora, the boundary zone between the *Eisenia* algal forest and the crustose coralline algal area is considered to be a substantial site for larval settlement and spat growing.

LARVAL DEVELOPMENT OF *HALIOTIS ASININA* LINNAEUS. **S. Sawatpeera,[2] E. Suchart Upatham,[2] M. Kruatrachue,[1] Y. P. Chitramvong,[1] P. Sonchaeng,[1] T. Pumthong,[1]** and **J. Nugranad,[1]** [1]Department of Biology, Faculty of Science, Mahidol University, Bangkok 10400, [2]Faculty of Science, Burapha University, Chonburi 20131, Thailand.

The larval development of *Haliotis asinina* was observed from fertilization to the formation of the fourth tubules of cephalic tentacle under water temperatures of 25, 28, 31, and 34 °C. The larvae had 42 stages of development. The time period for larval development depended on the water temperature, lasting 65, 49, 41, and 41 hours at water temperatures of 25, 28, 31 and 34 °C, respectively. After settlement, the development of postlarvae through to the formation of the first respiratory pore was observed under room temperature (28–35 °C). The velum was shed and the mantle began to secrete a new shell. Mouth, radula and digestive organs were developed on the third day after settlement. The heart was seen on the fourth day. A prominent structure apparent on the roof of the mantle cavity showed the formation of the ctenidium. The second pair of epipodium tentacles began to form and the eye stalks were completely developed by the eighth day after settlement. Between days 9 and 24, the postlarvae increased in shell size and number of epipodia and tubules on the cephalic tentacles. The ctenidium was more developed. The first respiratory pore began to form between days 24 and day 30, depending on the temperature, diatom type and larval density in the settlement tank.

EFFECT OF BIOFILM DENSITY ON GRAZING RATES OF *HALIOTIS FULGENS* POSTLARVAE. **R. Searcy Bernal, L. A. Vélez Espino,** and **C. Angniano Beltrán,** Instituto de Investigaciones Oceanológicas, Apartado Postal 453, Ensenada 22860, Baja California, México. (rsearcy@faro.ens.uabc.mx)

Grazing rates of *Haliotis fulgens* postlarvae of different ages (7, 15, 30, 45, and 60 days), feeding on the cultured diatom *Navicula incerta*, were estimated in 10 ml sterile plastic dishes, previously inoculated with different densities of the diatom (*ca.* 100-4 000 cells/mm^2). Postlarvae (3–7 per dish) were allowed to graze for 2–3 hours and video recordings were taken to estimate postlarval size and grazing rates by digital image analysis. Seawater was changed every second day and postlarvae were measured again after 6–8 days to estimate growth. Grazing and growth rates of postlarvae older than 15d increased linearly with biofilm density. The highest grazing rates for 7 and 60 day-old abalones were 79 and 10 999 cells/postlarva/hour, respectively. The most important increase in grazing activity occurred between ages 45d and 60d, when postlarvae reached 1.5–2.0 mm and started the formation of the first respiratory pore. Implications for the management of production systems are discussed.

ISOLATION AND CHARACTERIZATION OF MICROSATELLITE DNA MARKERS FOR THE TROPICAL ABALONE, *HALIOTIS ASININA*. **M. J. P. Selvamani, S. M. Degnan, D. Paetkau,** and **B. M. Degnan,** Department of Zoology and Entomology, University of Queensland, Brisbane, Australia.

In abalone aquaculture, marker assisted selection for growth would enhance industry development. Highly variable microsatellite DNA has been identified as a useful marker in assessing the level of genetic variation in a population. The high abundance and ubiquitous distribution of microsatellite loci in the genome make it also an appropriate marker for identifying quantitative trait loci and parentage and pedigrees. Microsatellites allow the parents of superior progeny to be identified in mixed family rearing environments, as is often the case in abalone, thus enabling selective breeding in commercial aquaculture farms. This study describes the isolation and characterisation of a number of highly polymorphic microsatellite loci in the tropical abalone, *Haliotis asinina* and their utility in identifying parents of individual juveniles. A partial genomic library of *H. asinina*, was screened for dinucleotide microsatellite DNA using a biotinylated $(AC)_{18}$ primer, cloned and sequenced. Out of 29 sequences containing microsatellites, 12 contained microsatellite motifs and priming sequences for detailed studies on the natural and cultured population. Using fluorescently labelled primers, PCR analysis of 30 individuals from Heron Island population demonstrated that 10 of the 12 loci are highly polymorphic with the number of alleles ranging from 7 to 15. The polymorphic loci were used to test the parentage of juveniles from a brood stock of three females and four males. These loci were also used to test their ability to amplify microsatellite loci in other species of abalone.

GROWTH OF JUVENILE ABALONE, *HALIOTIS FULGENS* PHILIPPI, FED WITH DIFFERENT DIETS. E. Serviere Zaragoza,[1] A. Mazariegos Villareal,[1] G. Ponce Díaz,[1,2] and **S. Montes Magallón,[3]** [1]Centro de Investigaciones Biológicas del Noroeste (CIBNOR). P.O. Box 128, La Paz, Baja California Sur. 23000. México, [2]CICIMAR, IPN, La Paz, B.C.S, [3]SEMARNAP. Delegación en Baja California Sur.

Growth rates of juvenile *Haliotis fulgens* (green abalone), 17.33 ± 2.13 mm shell length and 0.44 ± 0.16 body weight, were evaluated with five different diets over a period of 106 days. Three diets were based on algae, palm kelp *Eisenia arborea*, giant kelp *Macrocystis pyrifera*, and *Gelidium robustum;* one on seagrass *Phyllospadix torreyi*, and one was an artificial diet. Shell length and body weight growth rates varied between 0.0191 mm day^{-1} and 1.5 mg day^{-1} for *E. arborea* and between 0.046 mm day^{-1} and 5.5 mg day^{-1} for *M. pyrifera*. Higher specific growth rates (SGR) in length and weight were determined for *M. pyrifera:* 0.23 and 0.71% day^{-1} and for the artificial diet: .22 and 0.67% day^{-1}. Significant differences between these percentages and the rest of the diets were found. The highest mortality (11%) was in juvenile fed with the red alga *G. robustum*. Factors affecting abalone growth are discussed with special reference to protein percentage of the diets.

A CHRONICLE OF COLLAPSE: THE DYNAMICS OF TWO OVERFISHED GREENLIP ABALONE POPULATIONS. S. A. Shepherd and **K. R. Rodda,** South Australian Research and Development Institute, PO Box 120 Henley Beach, South Australia.

Two populations of greenlip abalone (*Haliotis laevigata*) in Backstairs Passage and Avoid Bay respectively collapsed over two decades of fishing. Annual surveys of the populations over >10 years during the period of collapse showed that in open habitats, recruitment failed when adult densities fell below about 0.2 m^{-2}, whereas at sites in bays or behind headlands recruitment failed more slowly. The differential failure of recruitment at both sites led to strong spatial contraction of the two metapopulations to remnant sites of higher larval retention. Stock-recruitment curves for the populations were mainly of the Beverton-Holt form and showed weak density-dependence. As population densities declined, recruitment variability increased, making the populations even more vulnerable to overfishing. Estimates of Z, the total fishing mortality rate, derived by ageing catch samples and doing a catch-curve analysis, showed little change during the decline and collapse of the populations. Furthermore, fishing effort declined concomitantly with the catch, indicating that divers respond to declining abundance by reducing effort. However, this behaviour is not sufficient per se to allow population recovery, which may be achievable only by extraordinary management measures such as closure.

SERIAL DECLINE OF THE SOUTH AUSTRALIAN GREENLIP AND BLACKLIP ABALONE FISHERY: TIME FOR A REQUIEM MASS OR A REVIVAL HYMN? S. A. Shepherd and **K. M. Rodda,** South Australian Research and Development Institute, PO Box 120, Henley Beach 5022, South Australia.

Historical catch information over two decades at the scale of the metapopulation show that 19 out of 45 metapopulations of the greenlip abalone (*Haliotis laevigata*) and 9 out of 45 putative metapopulations of the blacklip abalone (*Haliotis rubra*) have collapsed or seriously declined in the Western Zone of the fishery. In the case of greenlip abalone, populations of low initial productivity tended to decline at a faster rate than ones with high productivity, in accordance with the model of Shepherd and Baker (1998), and those on open rocky bottoms of low relief faster than those around islands or in bays. In the case of blacklip abalone, no pattern of decline was evident, except that sites of blacklip decline were also those where greenlip abalone declined. Fishing intensity was higher on populations close to home ports than on more distant populations. Fishing effort declined as the populations declined—not enough to allow the populations to recover, rather, of a sufficient intensity to ensure that they would not recover. As catches of inshore greenlip populations declined, fishing effort increased on more distant populations and has already caused the decline in density of adults to levels that presage recruitment decline, unless prompt action by management is taken to reduce fishing effort.

Management of multiple populations of two abalone species subject to different vulnerabilities and different intensities of fishing requires efficient detection of, and rapid response to, overfishing at the metapopulation scale. To facilitate focused research and timely management responses, we propose five fishery indicators as warning lights to be applied to every metapopulation; each indicator triggers an appropriate and unequivocal response of increasing management attention and/or severity. The response to all five triggered warning lights is to close the population in question to fishing and establish a recovery plan. For effective management, industry and management would need to agree in advance on the indicators and the designated responses. The appropriate indicators are certain to be species-specific and imply a good understanding of the species' population biology.

PARTIAL AND TOTAL SUBSTITUTION OF FISHMEAL WITH PLANT PROTEIN CONCENTRATES IN FORMULATED DIETS FOR THE SOUTH AFRICAN ABALONE, *HALIOTIS MIDAE*. **T. A. Shipton** and **P. J. Britz,** Department of Ichthyology and Fisheries Science, Rhodes University, PO Box 94, Grahamstown 6140, South Africa.

As proteins are the most expensive constituents in abalone feeds, it is necessary to evaluate them to produce least cost diets. Sixteen diets were formulated to contain 34% protein and 6% lipid and fed to juvenile abalone (initial shell length: 10.6 ± 0.1 mm). Dietary fishmeal was substituted at 30, 50, 75, or 100% with plant protein concentrates, and the growth and nutritional parameters recorded over a 180 day growth period. No significant differences were found in the growth rates between the control diet (100% fishmeal) and diets in which 30% of the fishmeal component had been replaced by either soya or sunflower meals, or torula yeast ($P > 0.05$). 50% fishmeal substitutions with either soya meal or spirulina did not effect growth rates ($P > 0.05$). Replacement of either 75 or 100% of the fishmeal with plant protein sources had a significant affect on growth ($P < 0.05$). Pearson product moment correlations between dietary lysine levels and either growth rates or protein efficiency ratios revealed positive correlations ($r = 0.77$, $P = 0.0005$; $r = 0.52$, $P = 0.04$ respectively), suggesting that lysine may have been the first limiting amino acid in these diets. Carcass analysis revealed that dietary protein source had no significant effect on body composition ($P > 0.05$).

PROTEIN CONTENT DETERMINES THE NUTRITIONAL VALUE OF THE SEAWEED *ULVA LACTUCA* FOR THE ABALONE *HALIOTIS TUBERCULATA*, *H. DISCUS HANNAI*, AND *H. FULGENS*. **M. Shpigel, I. Lupatsch,** and **A. Neori,** Israel Oceanographic and Limnological Research, National Center for Mariculture, P.O. Box 1212, Eilat 88112, Israel, and **N. L. C. Ragg,** Department of Zoology, University of Canterbury, Private Bag 4800, Christchurch, New Zealand.

The nutritional value to abalone of *Ulva lactuca* L. with different tissue nitrogen levels was studied. The seaweed was cultured at two levels of ammonia-N enrichment. Cultures receiving 0.5 g ammonia-N $m^{-2}d^{-1}$ ("Low-N") yielded 164 g $m^{-2}d^{-1}$ of fresh thalli containing 12% crude protein in dry matter and 12 kJ g^{-1} energy; cultures receiving 10 g ammonia-N $m^{-2}d^{-1}$ ("High-N") produced 105 g of fresh thalli $m^{-2}d^{-1}$ containing 44% protein and 16 kJ g^{-1} energy. High-N and Low-N algae, and a "standard" mixed diet of 75% *U. lactuca* and 25% *Gracilaria conferta* (w/w) containing 33% protein and 15 kJ g^{-1} energy, were fed to juvenile (0.7–2.1 g) and adult (6.9–19.6 g) *Haliotis tuberculata*, *H. discus hannai*, and *H. fulgens* in a 16 week feeding trial. Voluntary feed intakes of the High-N and standard diets were significantly lower than the Low-N diet in all the cases. Clear differences in performance between treatments were found in the juvenile and adult abalone of both species. Juveniles fed High-N and standard diets grew significantly faster (specific growth rate of *H. tuberculata* was 1.03% day^{-1} on High-N algae compared to 0.72% on Low-N algae; *H. discus hannai* grew 0.63% and 0.3% day^{-1} on High and Low-N algae, respectively) and showed much better food conversion ratios. The nutritional value of *Ulva lactuca* to abalone is greatly improved by a high protein content, attainable by culturing the seaweed with high supply rates of ammonia.

BIOCHEMICAL COMPOSITION OF BENTHIC MARINE DIATOMS USING AS CULTURED MEDIA A COMMON AGRICULTURAL FERTILIZER. **J. A. Simental Trinidad, M. P. Sánchez Saavedra,** and **J. G. Correa Reyes,** Aquaculture Department. Centro de Investigación Científica y de Educación Superior de Ensenada (C.I.C.E.S.E.). Apartado Postal 2732, Ensenada, Baja California, México. C.P 22800.

Three strains of benthic marine diatoms (*Navicula incerta*, *Navicula sp.* and *Amphora sp.*) were grown individually in batch systems with 10l of nonconventional culture medium formulated with three common agricultural fertilizers. The quantity and quality of the biomass produced with the nonconventional culture media were compared to those obtained with the traditional culture media "f/2" (Guillard and Rhyther 1962). The aim of the present work was to obtain a low price and alternative culture medium for benthic diatoms commonly used for commercial abalone culture. The quantity of biomass produced did not differ as a result of medium for each diatom culture. The general trends in biochemical composition evaluated as protein, carbohydrates and lipids content of each diatom culture showed, as expected, significant differences through time (10 days). The biochemical composition of *Amphora sp.* and *N. incerta* did not differ as result of the alternative medium, but the culture of *Navicula sp.* had significantly higher values for protein and carbohydrate concentrations, in comparison with the traditional culture medium. We consider that the chemical composition of the non-conventional medium does not limit biomass production, however, depending on the culture age, the quality of the biomass used as food for abalone postlarvae can change. The savings, in terms of cost of chemicals, range between 80 and 90%.

A NOVEL MASS CULTURE SYSTEM FOR BENTHIC DIATOMS. **J. A. Simental Trinidad, M. P. Sánchez Saavedra, J. G. Correa Reyes,** and **N. Flores Acevedo,** Aquaculture Department. Centro de Investigación Científica y de Educación Superior de Ensenada (C.I.C.E.S.E.). Apartado Postal 2732, Ensenada, Baja California, México. C.P. 22800.

The production of benthic diatoms as food for abalone postlarvae is an important consideration in commercial abalone farms. Common problems in this field include the economic cost of bio-

mass production, biomass quantity and quality. World wide, several culture techniques are used for the production of benthic diatoms, each of which has different culture requirements. The aim of the present work was to offer a new system for the production of benthic diatoms. This innovative system was designed using a 20l circular white plastic bucket, covered with transparent polycarbonate plastic lid "Lexan", with two orifices through which aeration was supplied to allow air circulation and to avoid water condensation and temperature increases. The biomass of benthic diatoms produced with this novel system was compared with that from two other classic systems. We obtained a higher biomass production and a reduction in the culture time. Other important considerations are the lower requirements of culture volume, culture area and cost. This system was experimented with to produce *Navicula incerta* and *Amphora sp.* which are commonly used as food for abalone postlarvae.

MOLECULAR TOOLS FOR COMPLIANCE ENFORCEMENT—THE IDENTIFICATION OF SOUTHERN HEMISPHERE ABALONE SPECIES FROM ABALONE PRODUCTS. N. Sweijd,[1] B. Evans,[2,3] N. G. Elliott,[3] and P. Cook,[1] [1]Department of Zoology, University of Cape Town, South Africa, [2]School of Zoology, University of Tasmania, Australia, [3]CRC for Aquaculture, CSIRO Marine Laboratories, Hobart, Australia.

Abalone poaching remains a serious threat to the sustainable utilisation of abalone stocks and presents a difficult problem to resource managers and compliance officials alike. In South Africa, Australia, New Zealand, Mexico, the USA and Canada, abalone poaching continues to varying degrees with reported cases involving significant quantities of abalone in terms of biomass and value. Due to over-exploitation, many abalone stocks are in decline. In the USA, one species is commercially extinct and others are threatened, resulting in the closure of the commercial abalone fishery. Several major abalone producing countries have commercial and/or recreational fisheries for more than one abalone species. In many cases, the species distributions traverse regional and even international boundaries. Thus, different authorities have jurisdiction over a species' range, leading to different regulations (e.g., season and size) applying within that range. To complicate these issues, once the animals have been processed, morphological identification of the species might be impossible, thereby creating an opportunity for alleged poachers not only to dispute jurisdiction, but also species identity.

Of the approximately 25 exploited and potentially exploitable species globally, 11 occur within the waters of South Africa, New Zealand and Australia. The need for a non-morphological method to distinguish between these species has led to the development of a suite of molecular markers to footprint these species genetically. PCR-RFLP tests, the target between 125 and 300 bp fragments of the lysin gene and the mitochondrial cytochrome oxidase 1, cytochrome oxidase 2, and 16s RNA have been developed. In combination, these markers can consistently distinguish between *Haliotis midae, H. spadicea* (South Africa), *H. rubra, H. conicopora, H. roei, H. laeviagata, H. scalaris, H. assinina* (Australia), *H. iris, H. australis*, and *H. virginia* (New Zealand). The development of these markers will contribute to more effective compliance enforcement. The conserved nature of the genes selected allows for further expansions of this approach to include all exploited abalone species in order to aid compliance in an increasingly global abalone market.

STUDIES ON PHAGE CONTROL OF PUSTULE DISEASE IN ABALONE *HALIOTIS DISCUS HANNAI*. L. Tai-wu, Marine Biotechnology Key Laboratory, Ningbo University, Ningbo, 315211, China, **J. Xiang** and **R. Liu,** Institute of Oceanology, The Chinese Academy of Sciences, Qingdao, 266071, China.

The isolation, purification and propagation of the phage of *Vibrio fluvialis*-II were studied. The phage was isolated from 12 different water samples, using the normal isolation procedure. The *Vibrio fluvialis*-II can grow well on STA (seawater, tryptone and agar) medium, which was used as a host and test culture. The plaques were obtained by the agar bilayer method. Concentrated phage suspensions were obtained from plates by washing them with 2% NaCl solution (e.g., add 4ml 2% NaCl solution into each plate); they were then put into a refrigerator at 4 °C overnight. The phage suspensions from the plate were added into a 5ml tube. The host bacteria were removed by centrifugation at 8000r/min, filtered and then purified and propagated by picking the single plaque repeatedly. The bacteria *Vibrio fluvialis*-II can be split using the phage at a high concentration. The effect of phage controlled pustule disease of abalone by muscular injection and infection of the wound in the abalone foot can raise abalone survival rate by up to 50%. Electron microscopic examination of the material taken from the plaques of the phage show that they contained simultaneously two forms of phage particle, with large and small heads, while their tails were morphologically identical. Numerous successive passages of the material taken from a single plaque did not allow the separation of small and large phage particles.

MORPHOLOGICAL CHANGES IN THE RADULA OF ABALONE, *HALIOTIS DISCUS HANNAI* AND *HALIOTIS IRIS*, IN RELATION TO THE TRANSITIONS IN THEIR FEEDING. H. Takami,[1] T. Kawamura,[1] R. D. Roberts,[2] and Y. Yamashita,[1] [1]Tohoku National Fisheries Research Institute, 3-27-5 Shinhama, Shiogama, Miyagi 985-0001, Japan, [2]Cawthron Institute, Private Bag 2, Nelson, New Zealand.

The radula morphology of *Haliotis discus hannai* and *H. iris* were examined by SEM from larval to adult stages. The overall length of the radula increased linearly with shell length (SL) in

both species. The radula of competent *H. iris* larvae (160 days old) contained ~10 transverse rows of teeth. The number of rows increased rapidly to 26–28 by 10 days post-settlement (533 ± 11 μm SL; mean ± SE). Six days post-settlement (458 ± 10 μm SL; mean ± SE), *H. discus hannai* had 20–26 rows. The number of rows remained at 25–30 throughout the remainder of the post-larval period (over ~500 μm SL), and started increasing again at ~4 mm SL. Radula width also increased linearly with shell length, due to an increase in the number of teeth per row and in the width of individual teeth. Marginal teeth were added steadily from just one pair per row in competent larvae to ~60–80 pairs per row in the adult. For both species, post-larvae <~1 mm SL contained only the two pairs of lateral teeth (L1, L2) present in the larval radula. An additional 3 pairs of lateral teeth (L3–L5) were added progressively between 0.9 and 1.9 mm SL in *H. discus hannai*, and between 1.0 and 1.7 mm in *H. iris*. The L3–L5 teeth became longer than the central teeth (R, L1–L2) as abalone grew above 1.5 mm SL, and the space between adjacent rows of teeth increased. Post-larvae <1 mm SL had highly curved rachidian and lateral teeth with clearance angles around or below zero, whereas larger post-larvae had positive clearance angles. These developments suggest that the teeth of post-larvae <1 mm SL function as "scoops" that slide across the surface, collecting small diatoms and fine, loose particles. The radulae of post-larvae <1 mm SL become more suitable for collecting larger particles and gouging feeding substrata. This pattern is consistent with the transitions in their feeding from microbial to macroalgal diets, and the improved ability of larger post-larvae to ingest large diatom cells.

AVAILABILITY AND DIETARY REQUIREMENTS FOR PHOSPHORUS IN JUVENILE ABALONE, *HALIOTIS DISCUS HANNAI* INO. B. Tan and **K. Mai**, Aquaculture Research Laboratory, College of Fisheries, Ocean University of Qingdao, Qingdao 266003, P. R. China.

Two experiments were performed to determine the availability and dietary requirements for phosphorus in juvenile abalone, *Haliotis discus hannai*. Abalone juveniles of similar size were distributed in a single-pass, flow-through system using a completely randomized design. Abalone were hand-fed once daily at 17:00, with the appropriate diets in excess. The feeding trials were run for 120 d. In Exp. I, the availability of phosphorus to juvenile abalone, *Haliotis discus hannai* from primary, secondary and tertiary calcium phosphate, primary sodium or potassium phosphate separately or in combination was determined in a 120-day feeding trial. The availability of dietary phosphorus was evaluated based on the growth rates of abalone, chemical analysis of the shells and soft bodies, and apparent absorption of dietary phosphorus from the digestive tract. The results indicated that among these inorganic compounds, primary calcium, potassium and sodium phosphates separately or in combination could be utilized effectively by juvenile abalone as dietary phosphorus sources (availability ranging from 87–97%). However, secondary and tertiary calcium phosphates were found to be low in availability, the values being 45% and 77%, respectively.

In Exp. II, five semi-purified diets were formulated to provide a series of graded levels of total dietary phosphorus (0.23–1.98%) from monobasic potassium phosphate (KH_2PO_4). The survival, soft-body to shell ratio (SB/S ratio), and calcium levels in whole-body (WB), soft-body (SB) and shell (S) were remained constant, regardless of dietary phosphorus level. However, the weight gain rate (WGR), daily increment in shell length (DISL), muscle RNA to DNA ratio (RNA/DNA ratio), carcass levels of lipid and protein, soft-body alkaline phosphatase (SBAKP), and phosphorus concentrations of WB, SB and S were significantly (ANOVA, $P < 0.05$) affected by the dietary phosphorus level. The dietary phosphorus requirement of the abalone was evaluated based on WGR, DISL, and RNA/DNA ratio respectively, by using the second-order polynomial regression analysis. Based on these criteria, about 1.0–1.2% total dietary phosphorus, i.e. 0.9–1.1% dietary available phosphorus, is recommended for the maximum growth of the abalone.

Zn AND Fe IN THE FORMS OF METHIONINE CHELATION OR SULPHATES AS SOURCES OF DIETARY MINERALS FOR JUVENILE ABALONE, *HALIOTIS DISCUS HANNAI* INO. K. Mai and **B. Tan**, Aquaculture Research Laboratory, College of Fisheries, Ocean University of Qingdao, Qingdao 266003, P. R. China.

Two feeding experiments were conducted to determine the dietary mineral (zinc and iron, respectively) requirements of juvenile abalone, *Haliotis discus hannai*, with amino chelated forms (zinc methionine and iron methionine, respectively) and inorganic forms (zinc sulfate and iron sulfate, respectively) as the mineral sources and to compare the bioavailability of the two forms of mineral, using a premium quality diet based on casein-gelatin as the protein sources. In Exp. I, 13 semipurified experimental diets containing graded levels of dietary zinc (5.6–84.6 mg zinc/kg) provided as either ZnMet or $ZnSO_4$ were fed to juvenile abalone in triplicate for 16 weeks. The results showed that the growth rate of the abalone, soft-body alkaline phosphatase activity and soft-body zinc concentrations were significantly (ANOVA, $P < 0.01$) affected by dietary treatment and responded in broken-line models to increases in dietary zinc levels from the two zinc sources. The requirements of dietary zinc as determined by broken-line regression analysis were: 16–18 mg/kg with ZnMet as the zinc source, and 35 mg/kg for $ZnSO_4$. This experiment also showed that the

bioavailability of dietary zinc with ZnMet as the zinc source is approximately 3 times as high as that with $ZnSO_4$ as the zinc source to juvenile abalone, *Haliotis discus hannai* Ino.

In Exp. II, experimental diets containing graded levels of dietary iron (24.9–212.7 mg iron/kg) provided as either FeMet or $FeSO_4$ were fed to juvenile abalone in triplicate for 16 weeks. Abalone fed the basal diet without iron supplementation exhibited significantly (ANOVA, $P < 0.05$) lower survival rates and carcass protein (%) than did the other groups. The growth rate and soft-body iron concentration of the abalone were significantly (ANOVA, $P < 0.01$) affected by dietary treatment and responded in broken-line models to increases in dietary iron levels from the two iron sources. The requirements for dietary iron determined by broken-line regression analysis, was recommended to be 65–70 mg/kg with either FeMet or $FeSO_4$ as the iron source. This experiment also showed that the bioavailability of dietary iron with $FeSO_4$ as the iron source is as high as that with FeMet as the iron source for juvenile abalone, *Haliotis discus hannai* Ino.

THE SOUTH AFRICAN ABALONE (*HALIOTIS MIDAE*) FISHERY: A DECADE OF CHALLENGES AND CHANGE. **R. J. Q. Tarr,** Marine and Coastal Management, Private Bag X2, Rogge Bay, Cape Town, South Africa.

The South African abalone *Haliotis midae* fishery is now 50 years old. The commercial sector has recently been consolidated into one category, rights holders, who may both dive and market their catch. Previously divers and processors were separate entities. A new overall Total Allowable Catch (TAC) has been initiated, which is 693t (whole mass in shell) for the 1999/2000 season. This includes a commercial TAC of 500t, a new "subsistence" TAC of 45t, and a recreational TAC of 148t. Poaching is a major problem, and large volumes are being taken, of which a high proportion are sublegal size abalone. This has resulted in serious reductions in TAC in one zone from 150t to 15t. In addition, movement of rock lobster *Jasus lalandii* into two of the four major TAC zones has interrupted the normal recruitment cycle of *H. midae* due to lobster predation on sea urchins *Parechinus angulosus*, on which juvenile abalone depend for protection.

Intensive fishery-independent diving surveys are underway. Co-management and ranching pilot projects have been initiated. Modelling of the resource dynamics per zone is being carried out using an age structured production model. Due to the combined effect of poaching and ecological changes, the prognosis for the resource is now poor, with future reductions in TAC likely.

SOUTH AFRICAN FISHERY INDEPENDENT ABALONE SURVEYS. **R. J. Q. Tarr, P. V. G. Williams, A. J. Mackenzie, E. Plaganyi,** and **C. Moloney,** Marine and Coastal Management, Private Bag X2, Rogge Bay, Cape Town, South Africa.

New fishery independent abalone surveys (FIAS) were initiated in 1995 to provide an unbiased index of relative abundance of abalone (*Haliotis midae*) for each fishery management zone. Density data from previous surveys were used to calculate the effect of varying transect length and transect numbers on the coefficient of variation (CV). Given constraints over available diving time, a survey design was chosen that would yield an expected coefficient of variation of 23 to 26%. This required twenty evenly spaced GPS-located diving stations to be surveyed annually per major fishing zone. Additional transects were swum in smaller fishing zones and marine reserves. The coastline of each fishing zone ranged from 15 km to 30 km in length. The transects are stratified in a 4m to 6m depth range, and each station comprises a 30m by 2m transect swum perpendicular to the coastline, by two divers. In addition, four "deep" stations (6 m water depth), with 50 m transects, are swum per zone. A total of 110 shallow and 14 deep stations are therefore swum each year.

Results have shown declining trends in abalone in all the major fishing zones since 1996. Variance has, in general, been within the predicted ranges. These data are being incorporated into age-structured models on which resource management decisions are based.

A PARAMETER ESTIMATION MODEL FOR GREENLIP ABALONE (*HALIOTIS LAEVIGATA*) POPULATION DYNAMICS. **N. A. Taylor (nee Dowling), R. McGarvey,** and **S. J. Hall,** School of Biological Sciences, The Flinders University of South Australia, G.P.O. Box 2100, Adelaide 5001, South Australia, Australia.

The degree of abalone aggregation has been found to be a key factor affecting greenlip (*Haliotis laevigata*) population sustainability. Larger aggregations contribute most to fertilisation success, yet are also most vulnerable to fishing. We have developed a deterministic model of the fishable population, structured with respect to aggregation size and incorporating a revised catch equation and matrix of re-aggregation probabilities.

The modified catch equation, which describes catch as a function of effort and catchability as a function of aggregation size, was derived from 1998 and 1999 field data. Catchability parameters were allowed to vary freely in the model and were fitted using historical catch by-numbers. The revised catch equation was incorporated into a cohort equation for abundance with an annual time step.

Recruitment (in terms of growth to the legal minimum length) was allocated equally among all cluster sizes, in accordance with results from field data, and was allowed to vary freely in the model. Fitted recruitment values suggested a high degree of density dependence among the fishable biomass, such that recruitment to the fishable biomass was suppressed when existing numbers were high.

Fitting the model to aggregation distributions from four years showed that recruitment alone did not adequately explain the observed shift into larger aggregation sizes in the absence of fishing. Re-aggregation was therefore incorporated in the model using a matrix of probabilities for individuals to move into any cluster size given their initial cluster size. The matrix was applied to the cohort equation output. Probabilities were assumed to follow a left-truncated generalised Poisson distribution.

This is the first model developed for abalone that is structured in terms of aggregation size. Moreover, the model formulation incorporates novel but realistic biological features that yield a better fit to empirical data. As such, this model provides a basis for assessing alternative management strategies in terms of their effect on a factor that has been shown to be critical for population sustainability.

SIMULATIONS OF RANDOM FISHING BEHAVIOUR AS AN INDEPENDENT TEST FOR ACTIVE TARGETING OF GREENLIP ABALONE (*HALIOTIS LAEVIGATA*) AGGREGATIONS. N. A. Taylor (nee Dowling), S. J. Hall, and **R. McGarvey,** School of Biological Sciences, The Flinders University of South Australia, G.P.O. Box 2100, Adelaide 5001, South Australia, Australia.

Aggregation patterns, analysed using multi-dimensional scaling analysis (MDS), and diver feedback, have indirectly suggested that divers actively target large (cluster size >20) aggregations of greenlip abalone (*Haliotis laevigata*). However, no independent analyses have been conducted to test the hypothesis that observed aggregation distributions may occur purely as a result of random fishing. We present the results of three Monte Carlo simulations of diver behaviour, where each simulation tested a different form of random fishing.

In the least random strategy, each individual abalone had an equal probability of capture, but once an individual was selected, the fishable proportion of the aggregation (cluster) in which it was found was also removed. Thus, larger aggregations had a higher probability of capture. The second, intermediate strategy assigned an equal probability of capture to the fishable proportion of each aggregation. The third, most random strategy ignored aggregation structure and randomly selected and removed individuals.

Aggregation data from field surveys conducted immediately prior to commercial fish-down experiments were used as input to the simulations. The output was compared, using Chi-Squared analyses, to the aggregation distributions from surveys undertaken immediately following the fish-downs. The results showed that the simulated aggregation distributions were significantly different to field survey distributions for all three strategies. We conclude that observed patterns in aggregation structure did not result from a random fishing strategy, and that divers do indeed target larger aggregations.

CLIMATE VARIABILITY, KELPS, AND THE SOUTHERN CALIFORNIA RED ABALONE FISHERY. M. J. Tegner, P. L. Haaker, K. L. Riser, and **L. I. Vilchis,** Scripps Institution of Oceanography, University of California, San Diego, La Jolla, California 92093-0201, USA.

Declines in landings in Southern California abalone fisheries and the eventual collapse of many stocks over the last two decades coincided with a period of greatly increased environmental variability. This included massive storms, an increase in the frequency of warm-water El Niño events after 1977, and an interdecadal-scale increase in sea surface temperatures. Kelp populations may be decimated by severe storms or warm water. Because of the strong inverse relationship between nitrate availability and water temperature, temperature is a good indicator of nitrate availability or stress; kelp growth ceases in warm nutrient-depleted water, tissue decays, and standing stocks may be greatly reduced. Abalones are directly affected by the availability of the drift kelp on which they feed, anomalously-warm temperatures may affect reproduction, and altered current patterns may affect larval dispersal. Because water temperature varies with location in Southern California and each of the five exploited species has its own thermal preferences, we chose to evaluate the role of environmental variability on red (*Haliotis rufescens*) abalone populations off three northern Channel Islands spanning a temperature gradient. We evaluate evidence for poor abalone growth and reproduction during El Niño events, water temperature anomalies, and monthly aerial survey data of giant kelp (*Macrocystis pyrifera*) canopies. The severity of El Niño disturbances and long-term changes in kelp standing stocks both correlated with the temperature gradient. Despite major long-term changes in kelp populations on the warmest island, the time scale of the decline in abalone landings predates the decline in kelps. The subsequent collapse of many populations, however, and especially the recovery of these depleted populations, may be directly related to kelp declines. Southern California abalones evolved in this disturbance regime, but the combination of extended periods of increased environmental variability with intense fishing pressure may have led to the loss of local populations, especially in warmer areas.

ABALONES AND SEA URCHINS: BIOLOGICAL AND FISHERIES INTERACTIONS. M. J. Tegner, Scripps Institution of Oceanography, University of California, San Diego, La Jolla, California 92093-0201, **K. A. Karpov** and **P. Kalvass,** California Department of Fish and Game, 19160 S Harbor Dr., Fort Bragg, California 95437.

Abalones and sea urchins share similar food and habitat preferences, and both are subject to fisheries. Here we contrast community changes under different fishing regimes in Southern and Northern California to consider the ecological interactions of red abalone (*Haliotis rufescens*), and red (*Strongylocentrotus franciscanus*) and purple (*S. Purpuratus*) sea urchins and their roles in kelp forest community structure. In Northern California, a well-managed recreational abalone fishery allows continued high abundances of red abalone. In contrast, a short-lived red sea urchin fishery dramatically reduced stocks of that species. In Southern California, abalone fishing was poorly regulated and many populations have collapsed, but higher rates of red urchin recruitment have sustained red urchin stocks. Purple sea urchin fishing is minimal in both areas, and these urchins may have been released from competition with red urchins and abalones. Here we compare changes in abundance and size-frequency distribution of these species in areas of each region open and closed to fishing, to evaluate the evidence for competition among the three grazers. Aerial photos of kelp canopies in Northern California during the period of intense red sea urchin removal strongly suggest increases in available food, as well as space, as adult abalones and purple sea urchins increased in abundance. We consider alternative explanations for these data, and discuss an ecosystem approach to management of these valuable resources.

ULTRASTRUCTURE OF NEUROSECRETORY CELLS IN THE CEREBRAL AND PLEUROPEDAL GANGLIA OF *HALIOTIS ASININA* LINNAEUS. A. Thongkukiatku, M. Kruatrachue, E. Suchart Upatham, P. Sobhon, C. Wanichanon, Y. Chitramvong, and **T. Pumthong,** Department of Biology, Faculty of Science, Mahidol University, Rama VI Road, Bangkok 10400, Thailand.

The ultrastructures of neurosecretory cells (NS_1, NS_2, and NS_3) in the cerebral and pleuro-pedal ganglia contained a euchromatic nucleus with a distinct nucleolus. The cytoplasm contained the usual organelles: RER, Golgi bodies, mitochondria and polyribosomes. There were two types of neurosecretory granules in the NS_1 of cerebral ganglia: type 1 were large osmiophilic membrane-bound granules and type 2 were small, electron-dense spherical granules. The NS_1 of the pleuro-pedal ganglion contained only one type of small electron-dense spherical granules. The NS_2 of the cerebral and pleuro-pedal ganglia had a nucleus that contained blocks of heterochromatin resembling a clock-face pattern. The cytoplasm contained the usual organelles, similar to those of NS_1. There was only one type of neurosecretory granule in the NS_2. In the cerebral ganglia, the NS_2 contained large membrane-bound granules with a crystalline structure. The NS_2 of the pleuro-pedal ganglion contained small electron-dense spherical granules. NS_3 were smaller than NS_1 and NS_2. The nucleus had a lace pattern of heterochromatin. There were fewer cytoplasmic organelles than in NS_1 and NS_2. The NS_3 of the cerebral and pleural ganglia contained similar neuro-secretory granules. They were composed of aggregates of dense osmiophilic globules of various sizes.

M. Tokley, Abalone Industry Association of South Australia Inc., 10 Alma Court, Flagstaff Hill, South Australia 5159.

The move from open access to limited entry, then to output controls and minimum size limits changed the way South Australian abalone divers operate to extract the Total Allowable Catch. This output/size limit control system used to manage the fishery to ensure sustainability of the resource, motivated divers to minimize their efforts and maximize catch efficiencies.

Industry divers restructured the "Le Mans" type race attitude and method of operating to a more mature and relaxed system with which to harvest abalone. In turn, the methods, techniques and periods during which abalone are harvested have also changed. Abalone divers concentrate on areas where catch per unit effort is maximized. This reduces the amount of time spent conducting fishing operations, which in turn, allows juveniles to replace the larger abalone taken and for stocks to re-aggregate to spawn. The fact that there is a quota and a minimum size limit at which abalone can be taken is sufficient to ensure that stocks are not decimated completely.

Industry believes that indices that can be used to measure the health of the stocks are CPUE, and the level of stock abundance of the fishery. Industry acknowledges stocks could be reduced to a level of eventual collapse, but only where size limits are not imposed to ensure next year's breeding stocks are available to help replenish those harvested. However, industry recognizes that there are areas in the fishery that were depleted through over exploitation during the open access years, and, up until the introduction of quotas, prevented divers from taking more than their fair share of the resource. These areas produce only ten per cent of the amount taken from the fishery each year, and thus will be left alone to recruit under natural means.

Industry has embarked on a program to relocate healthy abalone from good grounds to those grounds with low abundance and stunted abalone in the hope that they will reproduce and provide valuable breeding stock for future harvestable abalone populations.

FIRST CULTURE EXPERIENCES OF *HALIOTIS COCCINEA CANARIENSIS* IN A BIOFILTER SYSTEM. P. H. Toledo, R. Haroun, H. Fernández Palacios, M. Izquierdo, and **J. Peña,** Instituto Canario de Ciencias Marinas (ICCM), Ap. 56 35200, Teide, Las Palmas, Islas Canarias, Spain.

Haliotis coccinea canariensis is an endemic subspecies from the Canary Islands. Its highly appreciated flavour has led to it being overfished along the Canarian coasts. The present experiments were aimed to produce cultures of *H. coccinea canariensis* in an integrated biofilter system. Sixty abalone specimens of average shell length 4.2 cm were carefully collected from rocky shores by hand and scuba diving. After being sexed and marked, they were kept under natural light conditions in three indoor circular tanks of 2000 l and provided with constant seawater flux and aeration. They were fed with four species of algae: *Ulva rigida, Grateloupia dichotoma, Codium taylorii,* and *Cystoseira humilis,* produced in the biofilter system of the aquaculture experimental plant in the ICCM. Individual size (shell length and width) and weight were recorded on a monthly basis and spawning was induced in the late spring with U.V. light, seawater, hydrogen peroxide and TRIS solution. Abalone grew well with two of the selected algal species, namely *Ulva rigida* and *Grateloupia dichotoma,* although the former was better accepted and more efficient in promoting growth. Induced spawning was successful, and after two months of feeding with benthic diatoms, settled juveniles started feeding on macroalgae.

THE DIGESTIBILITY OF RAW, AUTOCLAVED AND PHYTASE TREATED LEGUMES IN GREENLIP ABALONE, *HALITOIS LAEVIGATA*. M. E. Vandepeer, P. W. Hone, R. J. van Barneveld, and **J. N. Havenhand,** SA Research and Development Institute Aquatic Sciences Centre, PO Box 120, Henley Beach, South Australia, 5022.

In this study we determined the apparent digestibility of nutrients in field peas, faba beans, yellow lupins and vetch in the greenlip abalone, *Haliotis laevigata,* and assessed whether autoclaving or the addition of phytase improved the digestibility of nutrients in these legumes. Fifteen isonitrogenous diets, consisting of one of three different treatments of the following legumes: field peas (*Pisum sativum*); yellow lupins (*Lupinus luteus*); faba beans (*Vicia faba*) and vetch (*Vicia sativa*), were formulated. The three treatments of each legume were raw, raw plus the enzyme phytase, and autoclaved. De-fatted soyflour served as a control. All legumes (whole seed) were ground in a hammer mill and included as the sole protein source of each diet. Each diet was fed to four different replicate tanks of 80–100 juvenile greenlip abalone, *Haliotis laevigata* (shell length 40–60 mm, 70 g wet weight). Faeces were collected each day by settlement and the apparent digestibility of gross energy, protein, amino acids and phosphorous in each diet was calculated using chromic oxide as the inert indicator (0.5 %). With respect to N digestibility for the untreated legumes, lupin > soyflour = beans > peas = vetch. Gross energy and dry matter digestibility had similar patterns, with lupin = soyflour > beans > peas = vetch. In general, autoclaving had a negative effect on digestibility, significantly decreasing the digestibility of all amino acids and protein from all legumes. Gross energy digestibility decreased for both soyflour and lupins, but increased for peas and vetch after autoclaving. The gross energy digestibility of beans was unchanged. The addition of phytase significantly increased the digestibility of phosphorous from the lupin diet only (84–91%). Strangely, the digestibility of phosphorous from pea diet actually decreased with the addition of phytase (94–87 %). In addition to its effect on phosphorous digestibility, increases in dry matter, nitrogen and the digestibility of some amino acids were observed with the addition of phytase.

THE EFFECT OF STARVATION ON GRAZING RATES OF *HALIOTIS FULGENS* POSTLARVAE. L. A. Vélez Espino, R. Searcy Bernal, and **C. Anguiano Beltrán,** Instituto de Investigaciones Oceanológicas, Apartado Postal 453, Ensenada 22860, Baja California, México.

Grazing rates of *Haliotis fulgens* postlarvae (30, 45, and 60 days old) after different periods of starvation (0, 1, 2, 3, and 4 days) were estimated by the digital analysis of video-recorded images of grazed areas. Trials were conducted in 10ml plastic dishes colonized by the cultured diatom *Navicula incerta* at standard densities (ca. 250–500 cells/mm^2). A significant increase in grazing rates at longer starvation periods was detected. However, for 45 and 60 day-old postlarvae, this increase occurred mostly during the first two days of starvation. The maximum grazing rates for 30, 45, and 60 day-old starved abalones were 3 861, 6 986, and 10 643 cells/postlarva/hour, respectively, which are similar to rates observed in parallel trials at much higher biofilm densities (ca. 4,000 cells/mm^2).

TETRAPLOID INDUCTION IN THE PACIFIC ABALONE *HALIOTIS DISCUS HANNAI* INO WITH 6-DMAP AND CB. G. Zhang, Z. Wang, Y. Chang, J. Song, J. Ding, Dalian Fisheries University, Key Laboratory of Mariculture Ecology, Ministry of Agriculture, Dalian, LN, 116023, PRC, **S. Zhan** and **X. Guo,** Haskin Shellfish Research Laboratory, Rutgers University, 6959 Miller Avenue, Port Norris, NJ, 08349, USA.

Triploid shellfish are useful in aquaculture because of their sterility, superior growth, improvement meat quality and sometimes increased disease resistance. The best way to produce trip-

loids is through diploid × tetraploid mating. The Pacific abalone, *Haliotis discus hannai* Ino, is a major aquaculture species in China. We studied tetraploid induction in this species by inhibiting the release of polar body I (PB1) with 6-dimethylaminopurine (6-DMAP) and cytochalasin B (CB). Gametes were obtained by inducing abalone to spawn with UV radiated seawater and artificially fertilized. Zygotes were treated with 6-DMAP and CB to block the release of PB1 and incubated at 22 °C. The 6-DMAP treatments, which were applied from 6min post-fertilization (PF) for 16–18min at concentrations of 175μM and 225μM, produced 20% and 22.5% of tetraploids, respectively, as determined by chromosome counts at trochophore stage (12–14 hours PF). Significant numbers of aneuploids (8.0–47.6%) were also observed. The majority of treated eggs (87.1–91.0%) survived to post-veliger stage (56–58 hours PF). Two CB treatments (0.8 mg/L) were applied to zygotes at 8 min PF lasting for 20 and 30 min. The short and long CB treatments produced 32.9% and 24.75% tetraploids respectively at trochophore stage, and 35.9% and 29.1% of tetraploid at post-veliger stage, respectively, as checked by FCM. Survival to post-veliger stage was 55.6% and 50.0% from the short and long CB treatments. The result suggests that CB is slightly more efficient than 6-DMAP for tetraploid induction in the Pacific abalone.

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ABSTRACTS OF PAPERS

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CONTENTS

THE BIOLOGY OF *PANDALUS*. Bo Bergström, The Royal Swedish Academy, Kristineberg Marine Research Station, S-450 34 Fiskebäckskil, Sweden.

The literature on the biology of 15 described species of the shrimp genus *Pandalus*, reported from the Pacific and Atlantic Oceans in the Northern Hemisphere is reviewed. Information on taxonomy, external morphology as well as species descriptions, keys to adult stages and systems for classification and species determination of larvae is treated. Present knowledge on geographic distributions, depth distributions and salinity, temperature, and habitat preferences on the continental shelves and slopes are also accounted for and discussed. Various aspects of the life histories of these species are described. The treatise on reproduction biology includes a discussion of evolutionary maintenance of protandric hermaphroditism, which is the dominating mode of reproduction in the genus, as well as an account of the present knowledge of the mechanism of sex change in these shrimp. Methods used to describe reproductive cycles as well as results are described and exemplified. Temperature effects on the duration of different phases of the reproductive cycles represented in the genus are discussed. Detailed accounts of adult behaviour in connection with mating, oviposition and hatching are presented. A section on *Pandalus* larvae includes accounts and discussions of behaviour, nutrition, growth and survival. Adult behaviour is treated both on individual and population levels, reports on schooling, migrations and food and feeding are reviewed and discussed. Predators, diseases and parasites on *Pandalus* are accounted for as well as some information on competitors. Methods for estimating growth and age and factors affecting growth are described and reviewed. Population dynamics, including population discrimination, abundance and standing stock estimation, parent stock-recruitment relationships and mortality factors, is discussed and illustrated by examples. Notes on the fishery on *Pandalus*, which from an economical point of view is surpassed only by the fishery for shrimp in the family Penaediae, and the potential for aquaculture concludes the review.

ASSESSMENT METHODS AND UTILIZATION OF SHRIMP STOCKS - FROM SIMPLE-MINDED APPROACHES THROUGH RESIGNATION TO MULTISPECIES AND SIMULATION METHODS. Gunnar Stefánsson, Marine Research Institute, Skulagata 4, P.O. Box 1390, 121-Reykjavik, Iceland.

Shrimp stock assessment methods have varied from simple bulk models through VPA approaches. The assumptions of such models typically fail miserably when shrimp stocks are considered. Recent years have seen some new methods for estimating population abundance and possible methods for utilizing resources difficult to assess. Such methods range from simulation of simple harvest control rules through very complex assessment models. Although these techniques still have a way to go, they represent promising ways forward.

PANDALID SHRIMP AS INDICATORS OF MARINE ECOLOGICAL REGIME SHIFT. Paul J. Anderson, National Marine Fisheries Service, Alaska Fisheries Science Center, Kodiak Laboratory, P. O. Box 1638, Kodiak, AK 99615-1638.

Pandalid shrimp are central components of the cold-regime boreal marine ecosystem in the Gulf of Alaska. Declines in abundance of several Pandalid species occurred quickly following water column warming due to an abrupt climate change after 1977. Shrimp trawl surveys conducted from 1953–1999 are used to describe how shrimp composition in catches changed relative to environmental parameters. Proportion of shrimp in survey catches was found to be negatively correlated with water column temperature. Pandalid shrimp species which occupied inshore and typically shallower water declined to near functional extinction, while offshore and deep water shrimp species have maintained low population levels. Possible mechanisms responsible for the chronic decline of several taxa of Pandalid shrimp and other crustaceans and replacement by other species are discussed. Abrupt climate change has an immediate effect on lower trophic levels of boreal marine ecosystems and rapid pandalid shrimp population changes are one of the first indicators that a community regime shift is underway.

SPATIAL SCALES OF SHRIMP (*PANDALUS JORDANI*) AGGREGRATIONS, ENVIRONMENTAL INFLUENCES, AND CONSEQUENCES FOR MANAGEMENT. R. Ian Perry and **J. A. Boutillier,** Pacific Biological Station, Fisheries and Oceans Canada, Nanaimo, B.C., Canada V9R 5K6.

Medium scale spatial patterns of smooth pink shrimp (*Pandalus jordani*) off the west coast of British Columbia exhibit periods of aggregation, and periods of dispersal. This affects catch rates by commercial vessels and affects the variance of survey estimates of abundance. Survey procedures for shrimp in this area have attempted to compensate for such changes in spatial pattern by using sector and spline interpolators over the area surveyed to estimate total abundance. Short-term availability of shrimp to commercial fishing gears is known to be influenced by environmental conditions such as tides, winds, and the amount of sunshine. In this study, we use commercial and survey data to identify spatial scales and patterns of shrimp aggregations and dispersal as influenced by environmental conditions and interannual variations in the abundance of shrimp. The goal is to improve understanding of the processes affecting the spatial distribution of shrimp so as to improve survey abundance estimates and management advice.

FORECASTING FISHERY PERFORMANCE FOR NORTHERN SHRIMP (*PANDALUS BOREALIS*) IN NAFO DIVISIONS 2HJ. Don G. Parsons, Northwest Atlantic Fisheries Centre, P.O. Box 5667, St. John's, Newfoundland, Canada A1C 5X1.

The physical environment is believed to have a major influence on northern shrimp (*Pandalus borealis*) populations. Decades ago, sea water temperatures from Boothbay Harbor were used to predict shrimp landings two years later in the Gulf of Maine. Recent, unpublished studies in eastern Canada showed that commercial catch rates for shrimp were negatively correlated with temperature (lagged several years) and that nitrate levels in surface layers might be useful to predict shrimp abundance.

In this paper, I use time-series analysis to construct predictive models for standardized catch rates (an abundance index) in a shrimp fishing area off the Labrador coast. Environmental data are incorporated as input series in transfer functions. Results support the hypothesis that cold conditions are favourable for shrimp at early life-history stages (larvae and juveniles). Model results fit the observed values well in most cases and catch rate predictions for several years are provided.

RELATIVE IMPORTANCE OF ENVIRONMENTAL AND ECOLOGICAL FACTORS TO THE MANAGEMENT OF THE NORTHERN SHRIMP (*PANDALUS BOREALIS*) FISHERY ON THE SCOTIAN SHELF. Peter A. Koeller, Department of Fisheries and Oceans, Bedford Institute of Oceanography, Dartmouth, Nova Scotia, Canada B2Y 4A2.

The biology of *P. borealis* on the Scotian Shelf is discussed in relation to the Gulf of Maine and Newfoundland Shelf stocks. The Scotian Shelf as a whole is an area of transition for *P. borealis* with a change in oceanographic characteristics in the mid shelf area that has resulted in populations with characteristics of both more southern and more northern stocks. The relatively large, commercially important population on the eastern Scotian Shelf is restricted to small areas of suitable habitat despite favorable temperatures over a wide area. On the southern and western Scotian Shelf a small population inhabits relatively small areas of marginally suitable temperatures, despite large areas of suitable habitat. Commercially important concentrations in this area appear only rarely after temperatures decrease to more favorable levels. Growth rates and age at first maturity on the Scotian Shelf are intermediate between the Gulf of Maine and the Newfoundland shelf, and migrations include inshore movements of ovigerous females in winter as in the Gulf of Maine, as well as localized ontogenetic migrations. While shrimp populations on the Scotian Shelf are influenced by water temperatures and habitat availability, predation pressure is also a significant determinant of abundance. The implications for the management of shrimp fisheries in the area are discussed.

PHYSICAL AND BIOLOGICAL FACTORS INFLUENCING RECRUITMENT OF NORTHERN SHRIMP *PANDALUS BOREALIS* IN THE GULF OF MAINE. Anne Richards, National Marine Fisheries Service, 166 Water St., Woods Hole, MA 02543, USA.

The *Pandalus borealis* population in the Gulf of Maine is at the southernmost limit of the species' distribution in the northwest Atlantic. Previous studies have shown that recruitment is related to both spawning biomass and spring surface temperatures (corresponding to the period of planktonic larval development). The purpose of this study was to extend these analyses by examining the influence of additional environmental factors (freshwater runoff, wind velocity and direction) and predation on recruitment. Runoff and wind patterns strongly affect circulation within the western Gulf of Maine and thus may affect recruitment through effects on retention and/or transport of larvae. Major perturbations in the predator community in the Gulf of Maine have occurred in recent decades due to overfishing, thus predation pressure on shrimp may have varied over time. Data on runoff and wind patterns were available from long term monitoring programs of the U.S. Geological Survey and NOAA's National Climatic Data Center. An extensive food habits database developed by the Northeast Fisheries Science Center (NEFSC, Woods Hole, MA) was used to identify primary predators of shrimp in the western Gulf of Maine. Aggregate abundance indices for the predators were developed from multispecies trawl surveys conducted by the NEFSC during 1968–1998. Shrimp recruitment was modelled as a function of shrimp spawning biomass, the environmental factors, and predator abundance at biologically appropriate lags.

HOW DOES THE PARTICULATE ORGANIC CARBON SEDIMENTATION WITHIN THE SEASONAL SEA-ICE REGIME INFLUENCE THE DISTRIBUTION OF NORTHERN SHRIMP (*PANDALUS BOREALIS*)? René O. Ramseier and C. Garrity, Microwave Group-Ottawa River, Inc.

Based on sediment trap data collected at a depth of 500 m below the ocean surface, as well as the *in situ* sea-ice regime, we have constructed a sedimentation model to map the amount and distribution of particulate organic carbon (POC) for the Greenland Sea (Ramseier et al. 1999). The derived model is based on ice regimes defined by: (1) ice concentration, (2) duration of ice cover and (3) distance from an ice edge, all relative to a trap location. In the case of POC the sedimentation can be determined using a mean annual ice concentration. For a severe ice year, the area of seasonal ice cover provides 89% of the POC sedimentation. In a light ice year the amount of POC is slightly reduced to 87%. To better understand the distribution of the POC sedimentation the model divides the seasonal ice cover into three distinct sub-regions, col-

lectively named the Biological Marginal Ice Zone (BMIZ). The Biological Marginal Ice Zone does not include all the seasonal ice cover extent, as would a Marginal Ice Zone. There is a centrally located sub-region within the BMIZ, where the sedimentation is non-linear resulting in a band of localized high sedimentation. This results in an elevated export of biologically produced particles to the deeper ocean. It is this result of localized sedimentation that is likely to affect the distribution of shrimp. As an initial test we have selected the Northern Shrimp Fishery area between 49°N and 60°N, Labrador Sea. Two data sets provided by Don Parsons (DFO), (1) commercial catches for 1989 and (2) research catches for Oct.–Dec. 1997, were analyzed in relation to the POC distribution based on mean annual seasonal sea-ice cover extent. Binning the commercial data according to POC, results in a correlation coefficient for a linear regression between catch per hour and POC of $r^2 = 0.926$. Similarly, binning the commercial data according to depth, results in a correlation coefficient of $r^2 = 0.995$. The research data on the other hand was binned according to (1) POC, (2) depth and (3) temperature with linear regression between total catch and POC. The correlation coefficients r^2 are (1) 0.535, (2) 0.897, and (3) 0.954 respectively. Analysis of the linear equations for the commercial fisheries catch per hour data, based on POC and depth binning, yield catch rates of 57.4% for POC, while depth accounts for 42.6%. For the research fisheries the relative contributions of the three variables in percent for the weight/shrimp versus POC give (1) 36.2, (2) 33.1, and (3) 30.7 respectively. The overall conclusion is that POC plays an important role as a food supply, and its distribution provides locations where to look for shrimp. Bibliography: Ramseier, R.O., C. Garrity, E. Bauerfeind, and R. Peinert, Sea-ice impact on long term particle flux in the Greenland Sea's Is Odden-Nordbukta region, 1985–1996, *J. Geophys. Res.*, 104:5329–5343, 1999.

WAS THE INCREASE IN SHRIMP BIOMASS ON THE NORTHEAST NEWFOUNDLAND SHELF A CONSEQUENCE OF A RELEASE IN PREDATION PRESSURE? **George R. Lilly** and **D. G. Parsons**, Science Branch, Department of Fisheries and Oceans, P. O. Box 5667, St. John's, Newfoundland, Canada A1C 5X1.

During the late-1980s and early-1990s the relative importance of groundfish and northern shrimp (*Pandalus borealis*) was reversed on the Northeast Newfoundland Shelf off southern Labrador and northeastern Newfoundland. The biomass of cod (*Gadus morhua*) and other groundfish decreased greatly whereas the biomass of northern shrimp increased both within the same area and in areas to the north. It has been suggested that the increase in shrimp was due to decreased predation. Temporal trends and biomass indices for shrimp and its predators were examined to determine if the timing of the changes is consistent with the above hypothesis. In addition, temporal changes in the quantity of shrimp consumed by the cod were determined from the catches of cod during autumn bottom-trawl surveys (1978–1996), analysis of the stomach contents of cod sampled during those surveys and the application of a consumption model for cod based on studies of gastric evacuation rate. Changes in consumption of shrimp by the cod were compared with changes in the shrimp population, with emphasis on determining whether the increase in recruitment to the shrimp population was coincident with a reduction in removal of young shrimp by the cod stock.

GROWTH PERFORMANCE, SIZE AND AGE AT MATURITY OF SHRIMP *PANDALUS BOREALIS* IN THE SVALBARD AREA RELATED TO ENVIRONMENTAL PARAMETERS. **Hege O. Hansen** and **M. Aschan**, Norwegian Institute of Fisheries and Aquaculture Ltd., (Fiskeriforskning), N-9291 Tromsø Norway.

The study area was the shelf slope northwest of Svalbard (north of 75°00′N) within a depth range of 200–600 m. Seven Subareas were considered for modal analyses (MIX 2.3) of *Pandalus borealis* length frequencies from 1992 to 1998. The L50 (carapace length where 50% are mature females) was calculated from a line fitted to the length frequency of each area. Differences in L50, growth and age at sex reversal were then related to environmental factors including temperature, stock density and fishing activity. The data show that the life history of shrimp in this area varies not only geographically, but also over time. Between six and nine year classes were identified in each area and age at sex change varied between five and seven years. Shrimp in the northern areas grew faster than in the south. Difference in growth and age at sex change were mainly explained by temperature variations. Areas dominated by cold polar water, north and south of Svalbard, and areas where Atlantic and polar water alternate and cause variation in the environmental conditions, show a slower growth and a higher age at sex change. The implications to stock assessments of this plasticity in the shrimp life history is discussed.

HYDROGRAPHIC CONDITIONS OFF EAST GREENLAND - THEIR POTENTIAL EFFECT ON THE DISTRIBUTION OF SHRIMP (*PANDALUS BOREALIS*). **Manfred Stein**, Institut für Seefischerei, Palmaille 9, D-22767 Hamburg, Germany.

Hydrographic conditions in the major shrimp catching areas north of 65°N off East Greenland were examined to determine

possible changes in the physical environment which might explain the southward shift of Shrimp aggregations observed from 1993 onwards. Based on the historic mean summer (JAS) hydrographic conditions in the area of the Denmark Strait, the paper outlines the regional distribution of Polar and Atlantic water masses on the Greenland-Iceland Ridge. It is shown that topographic features have a steering influence on the flow of these waters, and that they might be responsible for the entrainment of major shrimp aggregations. A salient topographic feature is the deep Kangerdlugsuak Fjord which is carved into the East Greenland Shelf, just southwest of the Dohrn Bank. Until 1992, the northern shrimp aggregations were confined to this fjord region. Recent hydrographic data as sampled during the German bottom trawl surveys off East Greenland, give a potential explanation for the observed southward shift of shrimp (*Pandalus borealis*) distribution from 1992 onwards. It is hypothesised that an increased advection of warm Atlantic water masses as observed during September 1993 and thereafter, led to a southward displacement of the shrimp concentrations, and hence to a southward shift of the catching areas. These "warm water conditions" are maintained through to the present. Sea surface anomaly data from the TOPEX/POSEIDON Satellite, show that the variability of sea surface elevation, which is mostly due to changes in the heat content in the upper water column of the area under investigation is in the order of about ten to twenty days.

SPATIAL VARIABILITY IN LENGTH FREQUENCY DISTRIBUTION AND GROWTH OF SHRIMP (*PANDALUS BOREALIS* KRØYER 1984) IN THE BARENTS SEA. Michaela Aschan, Norwegian Institute of Fisheries and Aquaculture Ltd., (Fiskeriforskning) N-9005 Tromsø, Norway.

The length frequency distributions (LFDs) of shrimp (*Pandalus borealis*, Krøyer 1984) in the Barents Sea varies both spatially and temporally. Survey stations were defined in four groups using correlation and cluster analysis. The groups were defined according to LFDs with many small shrimp comprising the first group and stations with the largest shrimp comprising the last. Distribution was depth dependent for the first three groups, while the largest shrimp occurred in all depths - their distribution may be determined by strong currents. Growth performance in any area requires a broad LFD including all length groups and presents a special challenge in sampling design. Cohort analysis of LFDs for 1992 to 1998 show that shrimp in the southern Barents Sea grow faster than in the north. On the basis of similar growth performance 14 sub-areas were defined. Further population analysis for each sub-area is difficult due to grate selectivity differences, especially in catch data. Only high resolution data should be selected when preparing input data for further analysis. Summation of the number of individuals by year class and area can then be used to run production models or multispecies virtual population analysis for the whole Barents Sea.

COD IMPACT ON THE STOCK DYNAMICS OF SHRIMP *PANDALUS BOREALIS* IN THE BARENTS SEA AND ITS APPLICATION IN MULTISPECIES MODELS. Boris I. Berenboim, A. V. Dolgov, V. A. Korzhev, and **N. A.Yaragina,** Knipovich Polar Research Institute of Marine Fisheries and Oceanography (PINRO), Knipovich Street, 6 Murmansk 183763, Russia.

Cod predation is considered to be one of the most important factors influencing shrimp population dynamics. Historical cod feeding studies indicate that in the years with high shrimp biomass their frequency of occurrence in cod stomachs increases. Quantitative cod feeding analyses from the joint Russian-Norwegian database indicate that shrimp became most important for cod feeding during spring-summer. Fish at age 3–6 had the maximum influence on shrimp stocks. In 1982–1997 shrimp biomass dynamics was correlated with cod abundance indices. Patterns of cod predation impact are used in calculating shrimp stock dynamics using multispecies VPA and production models.

BIOLOGY AND DISTRIBUTION OF *PANDALUS HYPSINOTUS* (BRANDT) IN THE NORTHERN PART OF THE SEA OF JAPAN. Alexey Buyanovsky, VNIRO, V. Krasnoselskaya, 17 Moscow 107120, Russia.

Seasonal observations of the *Pandalus hypsinotus* population in the Tatar Strait (46-49°N) were made in 1996–1998. Data include samples from 504 sites. Females release larvae in June. Oocytes develop from August to December. Fertilisation and spawning occur in winter and females bear the eggs for six months. However, females with outer eggs were present in samples through the whole year. Five age groups were distinguished from the samples which were collected in autumn 1998. Carapace lengths in the first group varied from 16 to 27 mm; second = 29–44 mm; third = 29–44 mm; fourth = 37–48 mm; fifth = 44–52 mm. The two first groups included 97% males, the third 100% transitionals, and the last 2 groups comprised 85% females with head row. The probable age of the first group is 2+. Males change their sex during the fifth year. The most significant growth occurs after the summer moult while the autumn and winter moults are not followed by growth. Analysis of latitudinal distribution allowed to distinguish three local populations in the eastern part of the strait. Dynamics of both densities and size structures of these populations indicate that they develop rather independently and large-scale migrations between them do not occur. Stock management should be based on quantitative estimations of each local population.

OBSERVATIONS ON THE BIOLOGY AND DISTRIBUTION OF NORTHERN SHRIMP, *PANDALUS BOREALIS*, IN THE GULF OF MAINE, FROM RESEARCH VESSEL SURVEYS. Stephen H. Clark, V. Silva, E. Holmes, and **J. B. O'Gorman,** Northeast Fisheries Science Center, National Marine Fisheries Service, Woods Hole, MA, USA.

Research vessel survey data collected since 1974 on northern shrimp (*Pandalus borealis*) in the Gulf of Maine provide useful insights on the biology of this species and on factors affecting its distribution. Length-frequency analysis confirms the basic patterns of growth and sex reversal determined by previous authors and also indicates that changes in demographic parameters occur at different population levels and under different environmental conditions. Spatial and temporal distribution patterns are strongly dependent upon temperature, depth, and substrate conditions, and different size/year classes tend to occupy different habitats.

OCCURRENCE OF VARIOUS SPECIES TAKEN AS BY-CATCH IN STRATIFIED-RANDOM TRAWL SURVEYS FOR SHRIMP (*PANDALUS BOREALIS*) IN NAFO SUBAREAS 0+1, 1988–98. Per Kanneworff and **D. M. Carlsson,** Greenland Institute of Natural Resources, Box 2151, DK-1016 Copenhagen K. Denmark.

Random-stratified trawl surveys for assessing the stock of shrimp (*Pandalus borealis*) have been carried out annually in the period 1988–98 in the shrimp distribution areas in NAFO Subarea 1 and a part of Div. 0A. By-catch has been recorded since 1991 by weight and number of species. A listing of all recorded by-catch species (or groups of species as determined on board) is given together with their prevalence. A preliminary analysis of annual and spatial variations of shrimp and selected by-catch fish species based on calculated densities is also presented.

DENSITY DEPENDENT SEX-REVERSAL IN PINK SHRIMP, *PANDALUS BOREALIS*, ON THE SCOTIAN SHELF. Peter Koeller, R. Mohn, and **M. Etter,** Bedford Institute of Oceanography, P. O. Box 1006, Dartmouth, Nova Scotia, Canada B2Y 4A2.

On the Scotian Shelf, transition of *Pandalus borealis* from male to female occurred at different sizes and ages, and could not be related to a minimum size, age, or number of instars. Our data do not show the positive relationship between density of older females and size at sex transition predicted by sex allocation theory. Size at transition was inversely related to density, which was attributed to density dependent growth affecting all stages. Density appears to become increasingly important as a factor determining growth as densities increase, while at low densities, other factors, including temperature, are also involved. We concluded that males have a growth related physiological threshold at which they are obligated to enter the transitional phase and that final maturation to the female is associated with a separate size related threshold. Transition is a growth phase that is extended in areas of early male maturation until the female threshold is attained. In warmer areas where the difference between male and female thresholds are great the benefits of protandry are decreased and the population may regress to the dioecious state, resulting in more primary females.

AN ASSOCIATION BETWEEN THE ANEMONE, *CRIBRINOPSIS FERNALDI*, AND THE SHRIMPS OF THE FAMILIES PANDALIDIAE AND HIPPOLYTIDAE. Bradley G. Stevens and **P. J. Anderson,** National Marine Fisheries Service, Alaska Fisheries Science Center, Kodiak Laboratory, P.O. Box 1638, Kodiak, AK 99615, USA.

A previously unrecognized association between the pink sea anemone *Cribrinopsis fernaldi* and several species of Caridean shrimp was observed and documented from a submersible at depths of 75–150 m in Kodiak, Alaska. Shrimp were aggregated in a radial pattern around anemones, beneath or just beyond the tentacle canopy. Species collected with a suction sampler included *Eualus suckleyi, Spirontocaris* sp., *Lebbeus grandimanus, L. groenlandicus,* and *Pandalus tridens,* but not *P. borealis* or *P. goniurus,* although they were probably also present. Numbers of shrimp per anemone increased with depth from 61 to 115 m, and more shrimp were observed on silty-sand than on sandy-gravel substrates. While associations between actinians and Hippolytid shrimp are common in tropical waters, this association is unusual because of its northern geographic location and the involvement of Pandalid shrimp.

PREDATOR-PREY RELATIONSHIP AND TROPHIC LEVELS OF THE PINK SHRIMP, *PANDALUS EOUS*, IN THE YAMATO BANK, THE SEA OF JAPAN. Takashi Minami, Japan Sea National Fisheries Research Institute, Suido-Cho 1-5939-22, Niigata, Japan 951-8121.

Pink shrimp *Pandalus eous* is an important commercial species in Japan and an important member of the deep-water ecosystem in the edge of continental shelf and isolated offshore banks such as the Yamato Bank (shallowest depth; 246 m) in the central part of the Sea of Japan. In the ecosystem, pink shrimp function as both predator and prey. An understanding of predator-prey relationships are essential in elucidating the position of the pink shrimp within food webs and the ecosystem. Diet composition of the pink shrimp were investigated and stomachs of the potential predators of the pink shrimp such as skate, sculpins, flatfish, cod, walleye pollock,

snailfish, lumpsucker, eelpouts collected in the Yamato Bank were analysed. Pink shrimp prey primarily consisted of small crustaceans groups such as Gammaridea, Euphausia, and Harpacticoida. Other common prey were Bivalvia, Caridea, and Polychaeta. Little dietary variation was seen with respect to predator size or depth. Dominant predators for pink shrimp were skate (*Bathyraja smirnovi*), eelpouts (*Lycodes tanakai*), *Allolepis hollandi*, *Petroschmidtia toyamaensis*, sculpin (*Maracocottus gibber*), and cod (*Gadus macrocephalus*). There was some evidence of cannibalism in pink shrimp. Trophic levels were estimated by means of stable isotope ratios for pink shrimp and other benthic animals collected in the Yamato Bank.

HYDROGRAPHICAL AND BIOLOGICAL PROCESSES OF IMPORTANCE IN DETERMINING RECRUITMENT VARIABILITY OF NORTHERN SHRIMP IN WEST GREENLAND WATERS. Soren A. Pedersen, Greenland Institute of Natural Resources, C/O Danish Institute for Fisheries Research, Dept. of Marine Ecology, Kavalergaarden 6, 2920 Charlottenlund, Denmark.

The poster presents preliminary results obtained from ongoing studies of recruitment processes of northern shrimp (*Pandalus borealis*) in West Greenland waters. Information on distribution and lipid composition of *Pandalus* shrimp larvae in relation to hydrography and potential food resources is presented. Ongoing studies with the objectives of 1) identifying the effect of hydrographic frontal regimes on larval and juvenile shrimp condition and survival potential and 2) investigating the potential use of tracer lipids to establish food web relationships are outlined and discussed.

PANDALID SHRIMPS OF THE BOREAL AREA: HISTORY OF FISHERIES AND RESEARCH WITH SPECIAL REFERENCE TO RUSSIA. Boris G. Ivanov, Russian Research Institute of Fisheries and Oceanography (VNIRO) 17, V.-Krasnoselskaya, Moscow 107140, Russia.

The history of research and management of Pandalid shrimp fisheries are described with special reference to Russia. All commercial pandalid species were described between 1814–1902. Hjort and Petersen discovered commercial densities of *Pandalus borealis* in Norwegian fjords in the late 19th century. A. Berkeley (1929,1930) discovered protandry in pandalids. By 1936–1941, *P. borealis* life history had been studied mainly in southern areas and it was concluded that the species has a similar life cycle everywhere. Rasmussen (1953) broke this assumption and demonstrated great variability in growth and maturation depending on local environmental conditions. Horsted and Smidt (1956) and Allen (1959) studied life history in the most and least severe areas. In Europe and North America the fishery for pandalids began in the late 19th century. The histories of the fisheries in European, American, and Japanese waters was described at the first International Pandalid Shrimp Symposium (Kodiak, Alaska, 1979) while that in Russia was poorly documented. In the North Atlantic USSR/Russia began to fish for *P. borealis* off West Greenland in 1974 but the introduction of the 200-mile zone in 1977 resulted in the migration of Soviet boats to the Barents Sea. By 1978 shrimp catches had reached 18,000 tons. In the Pacific Russia started shrimping (*P. hypsinotus, P. borealis*) in the Tartar Strait in 1979, off northeast Sakhalin in 1995, off south-west Kamchatka in 1996. After a 20-year hiatus shrimping also recommenced in the western Bering Sea. The historical fishery for shrimp was conducted in the Gulf of Alaska (*P. borealis*) and the Anadyr Gulf (*P. goniurus*).

DATA-POOR STOCK ASSESSMENT METHODS AND THEIR APPLICATION TO SHRIMP STOCKS. Robert Mohn, Marine Fish Division, Department of Fisheries and Oceans, P. O. Box 1006, Dartmouth, Nova Scotia, Canada B2Y 4A2.

Many stocks, especially invertebrates, do not have enough data to support traditional assessment methods. These stocks are candidates for so-called data poor methods. Recently, 1996–1998, ICES convened a Study Group (Study Group on the Assessment of Other Fish and Shellfish Species) to investigate data poor methods in the context of evaluating unassessed European stocks. The reports of this Study Group provide a catalogue of methods, approaches and software. A summary of their work, and that of other fora, is presented with emphasis on data needs, utility of output and applicability to shrimp stock data. The methods range from simple production models to heavily parameterized stock synthesis. Determination of the precautionary reference points is included. Some of the methods are applied to Scotian Shelf shrimp data as test cases.

ASSESSMENT OF *PANDALUS BOREALIS* STOCKS IN THE NORTHWEST ATLANTIC: CHALLENGES WITH CATCH AND CATCHABILITY. Steve Cadrin, National Marine Fisheries Service, 166 Water St., Woods Hole, MA 02543-1026, USA.

Lacking precise information on age and growth, some stock assessments of northern shrimp, *Pandalus borealis*, in the Northwest Atlantic have been based on models that integrate catch and stock size indices. The most commonly used models have been production models, such as ASPIC, and stage-based methods, such as the Collie-Sissenwine model. Both model types can provide estimates of uncertainty for stochastic risk assessment of management options. However, the absolute magnitude of stock size from either model can be misleading, especially when models are overparameterized, and catchability of surveys or fishing effort is poorly estimated. Simulations indicate that production models require a time series with a wide range of stock levels to provide reliable estimates of absolute stock size. Stage-based estimates of survey catchability are sensitive to natural mortality, relative sur-

vey selectivity of recruits, and the relative weight of observation errors. In assessments where estimates of catchability are judged to be unreliable, the results are often still useful for providing insights on trends in relative stock size and fishing mortality.

A NON-PARAMETRIC METHOD FOR ESTIMATING BIOMASS FROM TRAWL SURVEYS, WITH MONTE CARLO CONFIDENCE INTERVALS. **Geoff T. Evans, D. C. Orr, D. G. Parsons,** and **P. J. Veitch,** Northwest Atlantic Fisheries Centre, P. O. Box 5667, St. John's, Newfoundland, Canada A1C 5X1.

The probability distribution for biomass of many marine species varies in space, partly as a function of bottom depth. We describe a non-parametric method for using trawl survey data to estimate the probability distribution at any point in the survey region whose bottom depth is known. Integrating the expected value of the distribution over the region provides an estimate of the biomass in the region. Repeated resampling from the estimated distributions at the survey points enables us to compute a Monte Carlo confidence interval for the biomass. When we apply these methods to northern shrimp (*Pandalus borealis*) in NAFO Divisions 2HJ, we obtain confidence intervals that are narrower than those computed using methods based on random-stratified sampling and an assumed Gaussian distribution.

USE OF SUBJECTIVE PREDICTION IN OPTIMAL STRATIFIED SAMPLING WITH APPLICATION TO SHRIMP SURVEYS IN THE BARENTS SEA. **Alf Harbitz,** Norwegian Institute of Fisheries, And Aquaculture Ltd., N-9291 Tromso, Norway.

We consider stratified sampling and the task of applying subjective knowledge in predicting the number of trawl samples per stratum that minimises the cv of the abundance estimator. The constraint is a given vessel time available. It is assumed that the strata biomass means, arbitrarily scaled, are the only unknown parameters needed to find the optimal solution. The concept of a subjective prediction distribution of the unknown stratum means is introduced. The distribution is person-dependent and is determined based on intervals $[L,U]$ for the minimum and maximum subjectively predicted biomass values compared with the true measured values found after the predictions. The approach assumes a constant subjective confidence level defined as the probability of covering the true value in a random interval. A pilot subjective prediction experiment was conducted during the 1998 shrimp survey in the Barents Sea. Based on 62 $[L,U]$ predictions of shrimp biomass in the next trawl haul combined with the true biomass, the subjective prediction distribution for the cruise leader was estimated. The distribution was applied to her stratum predictions for the next survey. 10,000 random predictions of true strata means were simulated from the distribution. For each simulation cv-values of the abundance estimator were estimated based on relative strata means predicted from historical data as well as the subjective predictions. A significant cv-reduction was obtained based on a combination of subjective prediction and historical data, compared to the use of historical data alone.

A TRAWL SURVEY FOR *PANDALUS BOREALIS* IN WEST GREENLAND. **Dan Carlsson, O. Folmer, P. Kanneworff, M. Kingsley,** and **M. Pennington,** Pinngortitaleriffik Greenland Institute of Natural Resources, Nuuk, Greenland.

Stocks of northern pink shrimp *Pandalus borealis* have been assessed using CPUE data. Since 1988, a stratified random research trawl survey has also provided estimates of stock abundance, and on biological parameters of the resource. The survey covers the West Greenland fishing grounds between 150 m and 600 m deep, and occupies a research trawler for about 60 days. Stratification is based on depth, but strata are fairly small. Stations are allocated proportional to stratum area. Over time, the survey has changed. It has been extended into southern fishing grounds as the fishery developed there. Two-stage sampling was introduced to reduce the largest stratum standard errors, and a spline method was investigated for interpolation of additional stations. Gear changes have included replacing 44-mm stretch mesh in the liner with 20-mm since 1993, and using a trawleye to determine start position since 1997. Biomass estimates have had error CVs of the order of 15–30%, and have not been correlated with CPUE. In 1997 a group comprising science management, biologists, fishery advisers, and external survey expertise reviewed the survey design. The review suggested: abandoning 2-stage sampling; shortening the tows and increasing their number, and allocating more stations to the highest-yield group of strata. Making more tows would improve information about biological parameters, and shorter might improve biomass estimation. Catches in a small sample of ½-h tows in 1998 were no more variable than in 1-h tows, although an experimental survey in that year showed no correlation between contiguous 1-h tows and could not predict the effect of shorter tows. Changes suggested to the analytical methods included pooling the small design strata into 4 large groups based on depth, log transforming the data to reduce its skewdness, and smoothing the results to reduce year-to-year variation. Analysis suggests that the biomass has not varied much over the survey series. To implement some of these suggestions, a progressive shift toward ½-h tows has begun, and it is envisaged that some ½-h tows will be experimentally carried out as pairs of 15-min tows. The proportion of stations allocated to high-density strata has been increased. Buffered sampling has been used to control station placement, and to prevent stations from clustering within strata. A proportion of stations, randomly selected, has been fixed from year to year.

THE GULF OF MAINE NORTHERN SHRIMP FISHERY—A REVIEW OF THE RECORD. Stephen H. Clark, S. Cadrin, D. Schick, P. Diodati, M. Armstrong, and **D. McCarron,** Northeast Fisheries Science Center, National Marine Fisheries Service, Woods Hole, MA, USA.

The Gulf of Maine northern shrimp fishery has been a dynamic one, with landings varying greatly in response to resource and market conditions. A directed winter fishery developed in coastal waters in the late-1930s, which expanded to an offshore year round fishery in the late-1960s when landings peaked at over 12,000 mt in 1969. Landings subsequently declined to very low levels as recruitment deteriorated and the stock collapsed, precipitating closure of the fishery in 1978. The resource recovered under restrictive management and was relatively stable at low to moderate levels of exploitation into the 1990s, with 3–4 strong year classes recruiting to the fishery. In the mid-1990s, landings and fishing mortality increased sharply and abundance and recruitment have again declined. Environmental conditions have clearly played an important role in affecting survival and abundance, but fishing mortality has been the overriding factor since the late-1970s. Stock assessments have consistently shown that poor recruitment is more likely at low levels of spawning stock biomass owing to reduced total egg production. Under normal environmental conditions sustainable yields from the Gulf of Maine northern shrimp stock are probably about 3000–4000 mt per year.

ESTIMATION OF HARVEST RATES IN THE SPOT SHRIMP POT FISHERY IN SOUTHEAST ALASKA USING PRE- AND POST-FISHERY STOCK ASSESSMENT SURVEYS. John Clark, G. Bishop, and **T. Koeneman,** Alaska Department of Fish and Game, P. O. Box 240020, Douglas, AK 99824, USA.

The pot shrimp fishery in Southeast Alaska harvested an annual average of 355 metric tons of shrimp since the 1990/91 season with an average ex-vessel value of almost 2 million dollars (U. S.). Spot shrimp (*Pandalus platyceros*) comprise over 95% of the landed weight. Information on abundance, size and sex composition, distribution of spot shrimp populations and evaluation of the cumulative impact of fishing effort on the abundance and biology of this resource is essential to achieving an optimum sustainable harvest. Stock assessment surveys were conducted in limited areas two weeks prior to the fishery in 1997 and 1998 to collect this information. In February, 1999, a post-season survey was conducted approximately 4 months after the area was closed to evaluate the effects of the commercial fishery on the abundance and composition of the stocks and to determine if a harvest rate could be reliably estimated for spot shrimp stocks in two areas. A simple ratio estimator is used to estimate the harvest rate on spot shrimp in two adjacent areas. A more robust and informative abundance estimator based on the relative estimated abundance and size distribution of spot shrimp in the areas and selectivity of the 1 3/4 and 1 1/8 inch mesh pots is developed and applied to the 1997 and 1998/99 survey data. Confidence limits are estimated using a bootstrap approach for a random stratified sampling plan. Dockside samples, survey size distributions and residuals from the analysis are examined for evidence of high incidental mortality.

BY-CATCH REDUCTION IN AN OCEAN SHRIMP (*PANDALUS JORDANI*) TRAWL FROM A SIMPLE MODIFICATION TO THE TRAWL FOOTROPE. Robert W. Hannah, Oregon Department of Fish and Wildlife, 2040 SE Marine Science Drive, Newport, OR 97365, USA.

Two commonly used groundline designs in the ocean shrimp (*Pandalus jordani*) trawl fishery were fished side by side from a double-rigged vessel to compare catch rates of shrimp and bycatch. The designs compared were a traditional "tickler chain" groundline, which is shorter than, and runs below and in front of the fishing line of the trawl, and a ladder chain with a short roller section, set to run under and slightly behind the fishing line. Both nets were measured using a SIMRAD ITI trawl monitoring system to control for differences in net spread and rise. The ladder/roller groundline caught 64% fewer slender sole (*Eopsetta exilis*), 61% fewer greenstriped rockfish (*Sebastes elongatus*) and 33% fewer small rockfish (<8 cm total length) than the tickler chain groundline. However, only the difference in slender sole was statistically significant ($P < 0.05$) for these sample sizes in an ANOVA. After allowing for a wider net spread with the ladder/roller gear, catches of shrimp and other fish species were comparable for both gears. These results suggest that ocean shrimp trawls can be rigged to fish efficiently for shrimp and marketable fish with a groundline that runs behind the fishing line of the trawl. A test of an ocean shrimp trawl using dropper chains to maintain a constant height of the footrope above bottom, with no groundline at all, is recommended.

VARIATIONS IN THE GROWTH PATTERN OF NORTHERN SHRIMP (*PANDALUS BOREALIS*) IN THE GULF OF ST. LAWRENCE. Louise Savard, Maurice Lamontagne Institute, Department of Fisheries and Oceans, P. O. Box 1000, Mont-Joli, Québec, Canada G5H 3Z4.

Northern shrimp (*Pandalus borealis*) are found throughout the Estuary and the Northern Gulf of St. Lawrence in concentrations that sustain a commercial fishery that has landed more than 20,000 tons annually, for the last 3 years. The Estuary and the Gulf of St.Lawrence form a closed area which is divided into four fishery management units located from the west to the east. Bottom trawl research surveys have been conducted in these management units in August-September each year since 1990. Carapace length frequency distributions obtained from the surveys indicate that the mean size of the last mode of males, the length at sex reversal and the mean size of females have varied in two ways since 1990. The LFDs show a size gradient from east to west that was persistent

from year to year. However, similar size variations between years were also observed within each area. The size gradient indicates that the areas have a specific influence on growth but the similarity of the year to year variations suggests a synchronism in the events that cause the length variations. Hypothesis explaining these results as well as the impact of such variations on resource conservation and fishery management are discussed.

GENETIC CHARACTERIZATION OF THE NORTHERN SHRIMP *PANDALUS BOREALIS*, IN THE NORTHWEST ATLANTIC. Jean-Marie Sévigny, L. Savard, and **D. G. Parsons,** Ministère des Pèches et des Océans, Institut Maurice-Lamontagne, Mont-Joli, Québec, Canada G5H 3Z4.

Genetic variability of the northern shrimp, *Pandalus borealis*, in the Northwest Atlantic was studied at nine enzymatic loci. Samples were collected in seven regions of the Saguenay Fjord, the Estuary and the Gulf of St. Lawrence and in two regions off the Labrador-Newfoundland coast. Males, primiparous and multiparous females were sampled in each region in order to determine if gene frequencies within regions are temporally stable. Genetic distances between regions are low indicating that the level of gene flow may be high in this species. A cluster analysis of genetic distance did not reveal organization on the geographic scale of sampling. Furthermore, the interpretation of the population genetic structure of the northern shrimp is complicated by the fact that significant differences in allelic frequencies were observed among the maturity stages within some regions. This observation suggests that differences observed on the geographic scale between some of the studied regions may not be stable through time. Various hypotheses to explain these results are discussed.

FIXED STATIONS SURVEY FOR SHRIMP ABUNDANCE INDICES, 15 YEARS OF INVESTIGATIONS IN THE NORWEGIAN DEEPS AND SKAGERRAK. Stein Tveite, Institute of Marine Research, Flodevigen Marine Research Station, N-4817 His, Norway.

Since 1984 about 100 bottom trawl stations at fixed positions have been completed yearly in October-November. The catch of 0-group *Pandalus borealis* give an indication of year class strength, however, the catch of I-group gives a more reliable estimate compared to analytical methods and CPUE statistics. In this paper comparisons are made between variations in shrimp abundance and environmental factors such as fluxes of Atlantic water into the area, other hydrographic variations and abundance of fish species.

CATCHING JUVENILE NORTHERN SHRIMP (*PANDALUS BOREALIS*) IN THE ST. LAWRENCE ESTUARY WITH A RIGID FRAME TRAWL. Hugues Bouchard, J. Lambert, and **L. Savard,** Maurice Lamontagne Institute, Department of Fisheries and Oceans, 850 route de la mer, P. O. Box 1000, Mont-Joli, P.Q., Canada G5H 3Z4.

Data for stock status assessment of northern shrimp (*Pandalus borealis*) is traditionally obtained from commercial sampling and research surveys using a conventional bottom trawl at depths where small shrimp are rare. To make up for the lack of knowledge on the relative abundance of juvenile shrimp, a rigid frame trawl, 15 feet wide and 7 feet high, mounted on skates was developed at Maurice Lamontagne Institute with financial support from the shrimp fishing industry. The trawl was designed to be easily manipulated on a rear trawler of 65 feet in order to sample several stations in one day. The catch in the trawl should reliably represent the proportions of the different size-classes of shrimp present on the bottom sampled. The trawl is 67 feet in length. A liner of 9 mm-stretched mesh was added to the last 51 feet to retain small shrimp. During the research survey, the trawl was towed at about 2 knots for 20 min at depths varying from 50 to 260 m. The poster presented at the symposium deals with 3 main points: a) design of the rigid frame trawl with dimensions, b) method of fishing with the trawl, and c) handling the catch.

A NEW INTERPRETATION OF AGE-AT-LENGTH FOR SHRIMP (*PANDALUS BOREALIS*) IN DAVIS STRAIT AND INSHORE WEST GREENLAND WATERS. Dan M. Carlsson, Greenland Institute for Natural Resources, P.O. Box 2151, DK-016 Copenhagen K., Denmark.

Since the introduction of annual stratified-random trawl surveys in 1988 interpretation of age at length for the shrimp stock in West Greenland waters has been based on the age-length structure established by Savard et al. (1994) for shrimp in the Davis Strait using modal analysis of samples from 1982 to 1987. Survey samples from the inshore Disko area have shown similar modes as found in the offshore surveys from 1988 to 1997, and shrimp in the two areas have been considered to belong to the same stock and have been assessed as such. The progression from 1996 to 1997 of a distinct and significant mode of males in survey samples from the Disko area indicated that the old interpretation is not applicable in this area, even though reasonable results have been obtained with its use in recent years. Therefore, survey samples from both the offshore areas and the Disko area were reanalysed by modal analysis, and a new age-at-length structure derived, indicating that shrimp in both areas change sex from males to females at age 6 rather than at age 7. The new interpretation also shows distinct van Bertalanffy growth.

BIOLOGICAL AND ECONOMIC YIELD-PER-RECRUIT: ALTERNATIVE STRATEGIES FOR MANAGING PACIFIC OCEAN SHRIMP (*PANDALUS JORDANI*). **Charmaine M. Gallagher, R. Hannah,** and **G. Sylvia,** Oregon State University, Corvallis, Oregon, USA.

Selecting 'optimal' strategies for managing Pacific Ocean shrimp is challenging due to uncertain and variable natural mortality, recruitment, and growth. Although there are no explicit objectives for managing Oregon's shrimp fishery, managers have developed measures they believe will prevent long-term biological damage to the stock, protect age-1 shrimp from overharvesting and sustain long term fishery benefits. Developing harvest strategies such as mesh size and season dates are complicated by economic factors including differences in output prices as a function of shrimp size. To evaluate the potential importance of market price on shrimp management, this research uses equilibrium yield per recruit analysis to compare biological and revenue yield. The analysis is conducted over a range of natural mortality rates, for multiple age-at-entry dates, and fishing mortality rates. Growth is determined using length at age samples from the commercial catch. Results indicate that higher rates of natural mortality shift the biological and economic optimum toward younger shrimp and lower fishing mortality rates. Incorporating output prices may shift the optimum toward an older age of entry and a lower fishing mortality. For all analyses, the revenue generated at biological yield optimums is compared to revenue from maximizing economic yields. Future research will build on this analysis by including selectivity at length, variable recruitment, harvester and processor costs, and product quality.

THE UNCERTAINTY OF AN ASSESSMENT PROCEDURE FOR THE WEST GREENLAND STOCK OF *PANDALUS BOREALIS*. **Carsten Hvingel** and **Michael C. S. Kingsley,** Pingortitaleriffik, Greenland Natural Resources Institute, P. O. Box 570, DK-3900 Nuuk, Greenland.

Fishery resources are often assessed by standardizing catch performances of fleets over time, to create series of CPUE indices that are not affected by the way the fleets have changed, but which instead represent indices of biomass. Simple population dynamics models can be fitted to these series of yearly estimates of biomass index, along with the yearly landings. The shrimp resource off West Greenland was assessed by fitting separate models to four different fleets operating over different periods between 1976 and 1988. The four CPUE series were united by weighted least squares, and the fleet catch series were summed. A logistic model of population dynamics was then fitted to this data and a standard trawl survey series. It was difficult to know how reliable this complex three-stage process was, and a jack-knife procedure of leaving out one year's data at a time was adopted to find out. First we jack-knifed the entire procedure, leaving out, for each year in turn, all the data from commercial CPUE and surveys from all stages of the process. The results were: small scatter (CV 9.8%) in optimal fishing mortality, but larger and off-centre scatter in sustainable catch, ranging from 8.7% lower to 52.0% higher than the value based on all the data. Omission of the first year's data created an outlier with a 47.3% lower sustainable fishing mortality. The components of this variation were investigated by using all years' data as input to the standardized CPUE series, and then jack-knifing only the input to the logistic population model. The optimal fishing mortality varied about as much as before (CV 7.7%), but the sustainable catch varied much less and more symmetrically (from 8.5% lower to +9.2% higher). Much of the variation in estimated sustainable catch therefore appears due to uncertainty in the standardized CPUE series. However, omitting the first year's data from the input to the logistic population modeling phase continued to give an outlying point, with an MSYF that was 46.8% lower. The parameter estimates of the fitted population model therefore appear sensitive to characteristics of the first year's data.

AGE DETERMINATION OF NORTHERN SHRIMP, *PANDALUS BOREALIS*, IN ICELANDIC WATERS USING THE DEVIATION METHOD IN CONJUNCTION WITH THE METHOD OF MACDONALD AND PITCHER. **Unnur Skúladóttir,** Marine Research Institute, Skulagata 4, P. O. Box 1390, 121 Reykjavik, Iceland.

Aging of shrimp was carried out for 10 consecutive years in two very different areas in Icelandic waters - the Arnarfjordur fjord, and the offshore area north of Iceland. In the Arnarfjordur, it was not possible to interpret length frequency distributions of individual years with the technique of Macdonald and Pitcher i.e., Mix. Recruitment failed completely twice during the period so 2 year-classes were missing altogether in the series. It was therefore necessary to look at a series of years using deviations from the mean length frequency distribution of the 10 year series. By following the positive deviations, one age class could e.g., be followed for 7 years as a positive deviation. The mean lengths assessed from the deviations were then used as inputs for the mix method to calculate the proportions, new mean lengths for each age class for a given number of age classes. The offshore population was very different from the secluded fjord population. The former area had to be divided into many smaller areas in order to interpret age classes properly. Deviations calculated from a combined length frequency distribution accentuated peaks for each small area but combination before aging was not useful in this regard. Eventually mix was applied to length frequency distributions of every small area and the results combined and weighted by nominal catch of each small area to an overall catch number for the whole area per year. Up to 8 year-classes were detected.

THE TRAFFIC LIGHT: A COLOURFUL BUT UGLY APPROACH TO PRECAUTIONARY SHRIMP STOCK MANAGEMENT. P. Koeller Don G. Parsons, L. Savard, and **C. Fu,** Northwest Atlantic Fisheries Centre, P. O. Box 5667, St. John's, Newfoundland, Canada A1C 5X1.

At a recent meeting of the NAFO Scientific Council participants considered three example stocks for the application of precautionary methods, including one "data poor" stock i.e. shrimp (*P. borealis*) on Flemish Cap (NAFO Division 3M). For stocks such as 3M shrimp for which quantitative reference points, targets or limits could not be defined, Scientific Council endorsed the interim use of stock specific checklists which include multiple, qualitative indicators of resource status. This method, which is similar to the matrices used in Environmental Impact Assessment, uses the "Traffic Light" analogy because assessment results are categorized as "green," "yellow," or "red," corresponding to favorable, uncertain, or unfavorable stock conditions. Recent stock assessments for Atlantic Canadian shrimp stocks, including the Scotian Shelf, the Gulf of St. Lawrence and the Labrador-Newfoundland Shelf, were conducted using the Traffic Light/checklist approach and results were viewed positively by scientists (some), fisheries managers and industry. A major drawback of the method in its current form is that it does not link assessments to TACs or other management controls. Modeling results suggest that "Traffic Light" results could be linked to simple harvest control rules in a way that is consistent with shrimp stock dynamics and management requirements, creating an integrated management framework.

PACIFIC COAST SHRIMP TRAWL FISHERIES: NEW MANAGEMENT AND ASSESSMENT CO-MANAGEMENT PROGRAMS. Rick Harbo, L. Convey, J. Boutillier, and **D. Hay,** Department of Fisheries and Oceans, Operations Branch, Fisheries Management, Pacific Region, Nanaimo, B.C., Canada.

The diverse and complex Pacific shrimp trawl fishery takes place along the British Columbia coastline, in a number of small inshore areas and large offshore grounds. The fleet of 248 licences is a mix of beam and otter trawls. There are seven Pandalid species harvested commercially and fisheries vary in complexity from single to multiple species fisheries with a variety of markets, including machine-peeled, hand-peeled, frozen-at-sea, fresh, and live shrimp. Landings peaked at over 7300 tons, with annual landed values reaching $13.6 million. Landings have declined since 1996, to annual levels ranging from 2000 to 3000 tons at $5 to $7 million, due to low stock levels in offshore areas and more restrictive, precautionary management practices. Fishery management has developed rapidly from passive management at relatively low levels of efforts to a complex suite of management programs starting in 1997, including time and area closures, catch ceilings (arbitrary precautionary limits, historically based or forecasted) and quotas assigned to more than 30 new shrimp management areas. In-season area-swept trawl surveys using commercial and research vessels have led to fishery independent biomass indices and exploitation rates of 25–33% have been set initially. A long-term collaborative management and assessment program is being developed with stakeholders. It will include logbooks, catch monitoring, biological sampling, and fishery independent surveys. At-sea observers on board commercial vessels, research cruises and plant sampling have been undertaken to determine the catch composition and develop preliminary estimates of by-catch, with an emphasis on eulachon and halibut. There have been efforts in the fleet to develop gear improvements and a code of responsible fishing practices.

ANALYSES OF HARVEST STRATEGIES FOR PANDALID SHRIMP POPULATIONS. Caihong Fu, T. J. Quinn, and G. H. Kruse, School of Fisheries and Ocean Sciences, University of Alaska Fairbanks, 11120 Glacier Highway, Juneau AK 99801-8677, USA.

Pandalid shrimp species have unique life history features, for instance sex change, which could have great effects on population dynamics. These populations are also highly variable in annual recruitment, seasonal growth and natural mortality, which have profound influence on their dynamics. Our research was aimed at evaluating harvest strategies with these features and variations explicitly incorporated for achieving better management. In this paper, population dynamics were simulated over a 50-year timeframe. A constant harvest rate of 0.3 was imposed when the population level was above its threshold, and 0.0 when below the threshold. The following alternative harvest policies were evaluated: fishing right after hatching, fishing at the end of the growing season but before spawning, fishing right after spawning, reducing (or increasing) mesh size to increase (or reduce) catch of young shrimp, and imposing an area closure to protect ovigerous females. The policies were evaluated based on the fishing effort corresponding to the catch quota, probability of population going below threshold level over the 50 years, and annual recruitment success. The effectiveness of these policies was contingent upon seasonal and annual variations in growth and natural mortality. Recommendations are made on harvest policies corresponded to situations of various growth and natural mortality variations.

DEVELOPMENT OF A MANAGEMENT AND STOCK ASSESSMENT PROGRAM FOR THE POT SHRIMP FISHERY FOR *PANDALUS PLATYCEROS* IN SOUTHEASTERN ALASKA. Gretchen H. Bishop, T. M. Koeneman, and **C. A. Botelho,** Alaska Department of Fish and Game, Commercial Fisheries Management, and Development, P.O. Box 240020, Douglas, Alaska 99826-0020, USA.

The spot shrimp fishery in southeast Alaska is the last viable pot shrimp fishery in the state. *Pandalus platyceros* comprise 95% of the landed weight; the remainder is primarily *P. hypsinotus*. A

10-fold increase in participating vessels since 1960 has heightened concern for conservation and led to increasing restrictions, including limited entry in 1996. Vessel configuration is a function of market but appears to be evolving from small vessels with a limited fresh local market to larger catcher-processors, which sell frozen whole shrimp, primarily to the Japanese market. The season has shortened from year-round in 1981 to the current season which begins on October 1 and closes on February 28 or when the upper end of the guideline harvest level is reached, and opens again in districts with quota left on May 1. A mesh size of 1 3/4-in. and two categories of pot configuration, 'large' and 'small' with associated pot limits of 100 or 140 were defined in 1997. Guideline harvest levels (GHL's) for all districts were implemented in 1995 based upon long-term average catches. More active management, with in-season monitoring of catch began at this time. With increasing effort in this fishery we recognized a need to move towards a harvest rate management strategy, thus we began stock assessment of a limited area southwest of Prince of Wales Island (district 3) in 1997. We have established a survey protocol and collected baseline data on catch rate index of abundance of *P. platyceros* for 1997, and before and after the fishery in 1998/99. There is no consistent trend in shrimp carapace length, number per pot, or size at 50 percent female (L50) between 1997 and 1998. Pre and post fishery length frequency and catch per pot data are modeled using change in ratio techniques to attempt to estimate harvest rate, as described in a separate paper. We examined length frequency and L50 data for 1997 and 1998 and used Areview GIS to map harvest information from 1960–1997 in order to detect evidence of serial depletion. There was evidence of reduced harvest in 1997 and a significant decrease in average carapace length in 1998 for some sub-districts of district 1.

FIXED ESCAPEMENT: AN ALTERNATIVE TO QUOTA MANAGEMENT IN A SHRIMP FISHERY. Jennifer A. Bond and **J. A. Boutillier,** Department of Fisheries and Oceans, Pacific Biological Station, Hammond Bay Road, Nanaimo, B.C., Canada V9R 5K6.

Growth and recruitment overfishing in the spot prawn (*Pandalus platyceros*) trap fishery in British Columbia is managed using size limits and a fixed escapement system. The fixed escapement is implemented using an index of the number of spawners/trap caught in the commercial fishery. The development and application of this system is reviewed, including a description of the theoretical basis for this method of management, problems with implementation, and the implementation procedure. The size limits were introduced in 1985 and implemented in 1988. Industry has recognised the benefits of the size limits and has recently suggested increases to take advantage of price differentials. The fixed escapement system was initially based on empirical survey data. To evaluate the efficacy of this management system a number of experimental management areas were developed. This paper reviews the present state of progress in the development of a more model-based rationale for this system using data gathered from one of these experimental management areas, Howe Sound. Howe Sound has been closely monitored as an experimental prawn management area since 1985. Data available for the area includes catch records from the commercial fishery, biological sampling and catch composition monitoring of the fishery, and detailed information from pre- and post-fishery research surveys carried out every year.

MODERN TRAWLING AND BY-CATCH REDUCING DEVICES IN THE NORTH ATLANTIC SHRIMP FISHERIES. Roger Larsen, Norwegian College of Fisheries Science, University of Tromso, 9037 Tromsø, Norway.

This presentation deals with by-catch related problems in trawling for *Pandalus borealis*, including recent developments in fish excluder devices. New techniques in sorting grates, and double and triple trawl methods are also covered.

MANAGEMENT OF THE CANADIAN SHRIMP FISHERY. John Angel, Canadian Association of Prawn Producers, P.O. Box 1C1, Head of St. Margaret's Bay, Nova Scotia, Canada B0J 1R0.

The Canadian northern shrimp fleet has been cited for its responsible fishing practices and for its success in resource conservation and sustainable economic development. The northern shrimp fishery has been managed conservatively since its inception with sustainable development being the guiding principle. Quotas increased slowly in the early years of the fishery and exploitation rates remain low. Canada implements an elaborate management scheme for northern shrimp with tight controls on quotas, vessels, gear, and landings, including 100% observer coverage in the offshore factory freezer fleet. The offshore fishery is rights-based with license holders held to strict Enterprise Allocations per company. The inshore quota is fished competitively among participating vessels with quota being assigned on a coastal community basis. Penalties and license sanctions are severe for violators. Great strides have been made in technological development including gear selectivity, bycatch control, size selection, etc. The fishery is based primarily on a single species, *Pandalus borealis* (northern pink shrimp) and takes place off eastern Canada from 49°N to approximately 63°N. A second species, *Pandalus montagui* (striped shrimp), is commercially less important and is fished in limited quantities. The commercial fishery began in 1978 and developed slowly until 1986 when catches began increasing. Since 1996, quotas have increased rapidly from some 36,000 mt in 1996 to approximately 90,000 mt in 1999. Scientists believe the shrimp resource to be comprised of a single stock or stock complex although there are differences in rates of growth and maturation across the geographic range of the species. These differences pro-

vide the basis for delineating assessment and management units. Current stock status, especially in the southern areas, is very favourable with high biomass of male and female components. The offshore fishery is prosecuted by thirteen offshore factory freezer trawlers. All of the offshore catch is processed and frozen on board as either cooked or raw product. The inshore fishery is prosecuted by some 350 vessels landing fresh product to shore plants for cooking and peeling. Major markets are Asia, Europe and the U.S.

GEAR TESTING IN THE NORTHERN SHRIMP FISHERY IN THE GULF OF MAINE TO IMPROVE SIZE SELECTIVITY, REDUCE BY-CATCH AND DECREASE PRODUCTION LOSS. Daniel F. Schick and **M. Brown,** Dept. Marine Resources, McKown Point, W. Boothbay Hbr., ME 04575, USA.

The Nordmore grate has greatly reduced finfish by-catch except for finfish of a similar size to shrimp. Also, the grate appears to have shifted the size selection for northern shrimp downward somewhat. Two studies of size selection for northern shrimp and finfish were conducted comparing small diamond mesh with 1-3/4 inch diamond mesh (commercial minimum mesh) in the cod end with and without the Nordmore grate and with square mesh of three sizes in the cod end behind the Nordmore grate. The square mesh was tested using both knotted and knotless twine. Initial tests with a double Nordmore grate showed reasonable release of small shrimp with a bar spacing of 1″ in the first grate and 7/16″ in the second grate. Trials with bar spaces of 1/4″ (6.4 mm), 5/16″ (7.9 mm), 3/8″ (9.6 mm), 7/16″ (11.1 mm) and 1/2″ (12.7 mm) in the second grate showed 1/2″ provided the best escapement of small shrimp, but allowed too many larger shrimp to escape. The 7/16″ bar spacing gave the best combination of retention of larger shrimp and release of small shrimp. Small finfish did not readily escape through the bars of the second grate. Cod end strengtheners of 6″ diamond and 6″ square mesh placed outside the shrimp cod end revealed no shift in the size selectivity for shrimp. Thus strengtheners of sufficient mesh size to prevent masking of cod end mesh may be permitted to prevent the loss of shrimp production through cod end splitting on haulback.

TRENDS IN PROCESSING AND MARKETING IN THE GULF OF MAINE SHRIMP FISHERY. Roland Hurtubise, Tang of the Sea, Inc., Standish, ME, USA.

This paper reviews the history of the Gulf of Maine shrimp fishery; with particular reference to trends in processing and marketing, based on the author's 39 years of involvement with northern shrimp. Operations have been greatly affected by biological factors including unpredictable trends in abundance and seasonal changes in product quality. Industry representatives played a major role in developing the management program, and in recent years industry involvement has remained strong. Final comments center around the importance of maintaining continued production of Gulf of Maine shrimp and the author's perspectives on options for achieving this goal.

THE ICELANDIC SHRIMP INDUSTRY. Petur Bjarneson, Fisheries Association of Iceland, Reyjavik, Iceland.

This talk deals with the development of the Icelandic shrimp industry, including a history of its expansion and the current state of processing and marketing. The industry developed slowly from 1935 to 1967 and then grew quickly during a period of decreasing prices. Out of necessity Nordic countries, including Iceland, began to cooperate in marketing activities and have a long history of experience in this area. Current challenges include decreasing local catches in a situation where catches are increasing elsewhere (e.g., Canada) and world trade of raw materials has reached unprecedented levels.

FINFISH BY-CATCH EFFECTS ON THE QUALITY OF OCEAN SHRIMP, *PANDALUS JORDANI*. Vicki H. Kutzikowski, R. Hannah, G. Sylvia, and **M. T. Morrissey,** Hatfield Marine Science Center, Oregon State University, 2040 Marine Science Dr., Newport, Oregon 97365, USA.

By-catch reduction devices (BRD's) have been used voluntarily in the ocean shrimp, Pandalus jordani, fishery off the Pacific Northwest United States to reduce by-catch and the associated labor and time costs. It has been hypothesized that by-catch may affect the quality of the shrimp by causing breakage. In this study, the effect of finfish bycatch on the quality of shrimp was evaluated. A double-rigged commercial shrimp vessel was chartered for test fishing. One net employed a Nordmore grate BRD and the other served as a control. By-catch was measured from each net. The shrimp catch was kept separate by side of gear and day of fishing and sampled throughout processing. Shrimp samples were collected at seven stages from the deck to finished product and evaluated for percent breakage by weight. At greater than 50% by-catch, there was a small (1–7%), but statistically significant ($P < 0.1$) decrease in breakage of shrimp in the excluder net for samples taken before placement in the hold, after being iced and raked, before being cooked and after the second shaker. After this point the differences became non-significant. The mean percent breakage increased from a mean 2.4% breakage before placement in the hold to a mean 18.0% breakage in the final product. Other factors such as the molt condition, count per pound, carapace length, phosphate treatment, moisture and microculture were examined and found to not influence breakage. Based on these findings, it is concluded that finfish by-catch does contribute to the breakage of ocean shrimp, however the impact is small relative to breakage caused by other handling procedures.

MONITORING THE SHRIMP TRAWL FISHERY IN BRITISH COLUMBIA. **Jason Clarke** and **W. E. L. Clayton,** Archipelago Marine Research Ltd., 200-525 Head St., Victoria BC V9A 5S1, Canada.

In 1997, the Department of Fisheries and Oceans, Canada (DFO) implemented a new management regime for the shrimp trawl fishery in British Columbia in response to concerns of increased fishing pressure and lack of fishery data. The BC coast was divided into Shrimp Management Areas (SMA), and catch ceilings for these areas were established. To support this change, shrimp license holders were required to fund a data collection program that included: (1) a hail reporting system to monitor vessel activity, (2) a logbook catch monitoring system to monitor catch by area and species, and (3) an offshore observer by-catch monitoring program to collect catch composition information for shrimp trawls. Since 1997, Archipelago Marine Research Ltd., an independent service agency, has been providing these data collection services on behalf of the Pacific Coast Shrimpers' Cooperative Association. The information collected is forwarded to DFO for use in the management of the shrimp trawl fishery.

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ABSTRACTS OF TECHNICAL PAPERS

Presented at the 20th Annual Meeting

MILFORD AQUACULTURE SEMINAR

Milford, Connecticut

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CONTENTS

OVERVIEW, 20TH MILFORD AQUACULTURE SEMINAR. **Walter J. Blogoslawski,** U.S. Department of Commerce, National Oceanic & Atmospheric Administration, National Marine Fisheries Service, Northeast Fisheries Science Center, Milford Laboratory, 212 Rogers Ave., Milford, CT 06460.

The 20th Anniversary Meeting of the Milford Aquaculture Seminar attracted 39 speakers who gave 42 presentations covering such topics as bivalve culture in Europe and New England, clam culture, *Vibrio parahaemolyticus* surveillance on shellfish beds, oyster and lobster diseases, transgenic species and genetic modification to prevent disease, harmful algal blooms, high school aquaculture curricula, blue mussel cultivation in the Northeast and Pacific Northwest, designs for recirculating fish culture systems, and diseases of cultured tautog. Over 160 attendees from the US, Canada and Great Britain met during the two-day conference to discuss recent problems of the aquaculture industry and suggest potential solutions. The cooperative spirit shared by the participants, many of whom compete against each other in a difficult business, was evident in the roundtable discussion.

Staff from 41 different aquaculture companies, scientists from 11 universities, teachers from two local high schools with aquaculture-vocational programs, and staff from 7 marine laboratories attended the seminar. Also, the Director of the National Marine Fisheries Service met many of the attendees and presented a luncheon address.

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MAXIMIZING EFFICIENCY OF HARD CLAM, *MERCENARIA MERCENARIA*, CULTURE IN AN EXPERIMENTAL-SCALE UPWELLER. **Craig L. Appleyard** and **Joseph T. DeAlteris**, Department of Fisheries, Animal and Veterinary Science, University of Rhode Island, Kingston, Rhode Island 02881.

Upwellers have proved to be extremely effective as bivalve nursery units and their use is steadily increasing in North America. The re-analysis of previous work by others suggested an asymptotic relationship between growth (% volume increase per 30 days) and chlorophyll-a effective flow rate (chlorophyll-a flux per unit biomass, $\mu g/min\ kg^{-1}$). The purpose of the study was to develop a relationship between flow rate, stocking density and growth in order to determine the flow rate and density that optimizes growth. Furthermore, the study was designed to investigate other significant environmental parameters influencing bivalve growth in an experimental-scale upweller system. Hard clams, *Mercenaria mercenaria*, were grown from ~2 mm (longest axis) to ~13 mm in a forced flow floating upweller from June 21 to August 19, 1999 in Point Judith Pond, Wakefield, Rhode Island. The flow rate and stocking density were varied in order to produce a chlorophyll-a effective flow rate range of 360 to 1500 μg/min per liter of clam volume, and growth and environmental parameters were measured semi-weekly. During the initial experimental period (June 21 to 24) an asymptotic relationship was observed between growth (%/day) and chlorophyll-a effective flow rate. The asymptotic relationship did not hold for the remainder of the experiment because ambient environmental conditions appeared to limit growth. Specifically, growth was linearly correlated with morning-dissolved oxygen. The relationship is strongest for the upper third of the fastest growing animals because the removal of the slower growing animals eliminates the effect of flow rate on growth.

EXPERIMENTAL EVIDENCE FOR PHYTIC ACID-PHOSPHORUS USE BY PURE CULTURES OF MARINE MICROALGAE. **Bethann Balazsi,** Natural Science Division, LIU, Southampton, NY 11968; **Gary H. Wikfors,** USDOC, NOAA, National Marine Fisheries Service, Northeast Fisheries Science Center, Milford Laboratory, Milford, CT 06460.

Phytic acid, or phytin, is a cyclic organic molecule containing six phosphorus atoms. Approximately half of the phosphorus in agricultural livestock feeds, e.g., corn and soybeans, is in the form of phytic acid. Most livestock animals are unable to digest phytic acid; therefore, essentially all phytic acid consumed is released as waste that may find its way into coastal waters. Some soil fungi and bacteria produce a phytase enzyme that breaks down phytic acid, thereby releasing the phosphorus in the oxidized form of phosphate ions. The capability of marine and estuarine microalgae to obtain phosphorus from phytic acid directly, without fungal or bacterial decomposition, has not been investigated systematically, although some reports have suggested that harmful dinoflagellates may be selectively fertilized by phytic acid in coastal waters polluted with livestock waste. In this study, we tested the capacity of eight bacteria-free microalgal strains, from several major algal classes, to grow on phosphorus from phytic acid.

Culture media (E medium basal) were prepared containing isomolar phosphorus (P) as either phosphate, phytic acid, or a 50:50 mix of the two, or with no added phosphorus source. Each of these media then was treated with commercially-available phytase enzyme (BASF Corp.) or left untreated, resulting in eight different medium treatments. Triplicate, 10-ml test tubes of each medium were inoculated with algal strains that had been grown for one subculture in medium with no added phosphate to deplete any cellular P reserves. Algae tested were: dinoflagellates, *Prorocentrum micans* (CCMP1589) and *P. minimum* (EXUV); diatoms, *Chaetoceros neogracile* (Chaet-B) and *Amphora coffeaeformis* (A-ORA), prymnesiophytes; *Isochrysis* sp. (T-ISO) and *Pavlova gyrans* (#93), and green algae; *Tetraselmis chui* (PLY429) and *Du-*

naliella tertiolecta (DE). Culture growth was monitored with thrice-weekly spectrophotometer readings, and final cell densities were determined by cell counts after 40 days.

Growth curves plotted from spectrophotometer readings showed at least some initial growth of all strains (with one exception) in all media, including media with no added P. This growth indicates that seawater used in the media contained some available P, or possibly that stored P in inoculum cells was sufficient to support limited cell division. This initial growth made maximal algal division rates unreliable for comparing experimental treatments; therefore, results were analyzed in terms of final population densities. *P. micans* did not grow in any medium, possibly because of lethal phosphorus starvation of the inoculum culture, and was eliminated from results analysis. Of the seven remaining algal strains, only two showed clear evidence of phytic acid-P use without phytase enzyme: T-ISO and A-ORA; neither of these is a dinoflagellate. Interestingly, it was not clear from statistical analysis if phytase enzyme effectively released P from phytic acid in a form usable by the algae, because addition of phytase enzyme alone (without phytic acid) consistently stimulated algal growth equal to the phytic acid + phytase treatment. The commercial phytase product is extracted from microbial biomass, and may contain phosphorus itself. These results indicate that microalgae other than dinoflagellates can possess the capacity to obtain phosphorus directly from phytic acid. This finding has application in management of coastal water quality, as well as in aquaculture, where phytic acid in cultured fish waste can be expected to support a more diverse algal assemblage than previously suggested.

THE IMPORTANCE OF TEMPORAL AND SPATIAL REPLICATION OF FIELD EXPERIMENTS: EFFECTS OF SEAGRASS COVER ON THE GROWTH AND SURVIVAL OF CULTURED JUVENILES OF THE SOFT-SHELL CLAM, *MYA ARENARIA*, AND HARD CLAM, *MERCENARIA MERCENARIA*. **Brian F. Beal**, University of Maine at Machias, 9 O'Brien Avenue, Machias, ME 04654.

Field experiments in marine soft-bottom habitats that are designed to test specific hypotheses about mechanisms regulating growth, survival, or fecundity of infaunal, hatchery-reared clams and quahogs are difficult to conduct due to logistical problems imposed by the nature of soft sediments.

These constraints mean that field trials typically suffer from poor replication (both spatially and temporally). Generalizations about processes that regulate these populations rarely can be made. For field culturists or communities seeking practical information concerning seed size, planting dates and locations, or what types of predator deterrent devices to use, the scientific literature provides conflicting lessons that relate directly to this inability to generalize.

Effects of submerged aquatic vegetation, such as eelgrass, *Zostera marina*, on the survival and growth of infaunal bivalves provides an interesting example for culturists to consider. The current paradigm states that grass beds provide spatial refuges for clams and quahogs. The mechanism for this protection has been shown experimentally to be related to the presence of the upright blades which affects visual predators, and, more importantly, the presence of below-ground roots and rhizomes which reduce the mobility and effectiveness of both infaunal and epifaunal predators.

From 1990–1999, five independent 3- to 6-month field tests using hatchery-reared juveniles of *Mya arenaria* (SL = 5 to 15 mm) were conducted on a tidal flat in Cutler, Maine to examine the influence of the presence or absence of eelgrass on clam growth and survival. From 1980-1981, similar field tests using cultured juveniles of *Mercenaria mercenaria* (SL = 8 to 15 mm) were conducted in two shallow subtidal regions of eastern North Carolina. Results from both geographic locations and across time generally discount and challenge the current paradigm. Survival rates of *M. arenaria* were significantly reduced in eelgrass beds compared to adjacent, unvegetated areas primarily due to increased predation by crabs and other crustacean predators. Similarly, grass beds in North Carolina afforded *M. mercenaria* no refuge from predators. Growth rates of *M. arenaria* were significantly depressed inside vs. outside eelgrass beds. Growth rates of *M. mercenaria* either showed no difference between habitats or were higher in grass beds. These studies indicate the importance of replicating field experiments temporally and spatially. Both public and private aquaculture programs should exercise caution about placing small bivalve seed into or near grass beds as they are habitats where crabs seek refuge from their predators (gulls, large fish, and other crustaceans).

PREY SIZE AND SPECIES SELECTION BY THE ASIATIC SHORE CRAB, *HEMIGRAPSUS SANGUINEUS* (DeHAAN) FEEDING ON BIVALVE PREY. **Diane J. Brousseau, Margot Gallowitsch**, and **Lenka Hurton**, Fairfield University, Fairfield, CT 06430.

Prey selection by the Asiatic shore crab *(Hemigrapsus sanguineus)* was investigated in relation to bivalve prey of different sizes and species. In the laboratory, four size classes of male crabs (Small, 10–15 mm CW; Medium, 15–20 mm CW; Large, 20–25 mm CW; X large, >25 mm CW) and three size classes of female crabs (Small, 10–15 mm CW; Medium, 15–20 mm CW; Large, 20–25 mm CW) were offered three bivalve species, *Mytilus edulis, Mya arenaria* and *Crassostrea virginica*. Ninety-eight percent of both the male and female crabs tested ate bivalve prey. Male crabs in all size classes selected larger *M. edulis* than similarly-sized female crabs, whereas medium and large male crabs selected larger

C. virginica than female crabs but only the largest male crabs selected larger *M. arenaria* than the females. Maximum prey sizes eaten by the Asiatic shore crab were *M. arenaria*, 20–25 mm SL; *M. edulis*, 15–20 mm SL and *C. virginica*, 10–15 mm SL. Both male and female crabs ate significantly more *M. arenaria* than either *M. edulis* or *C. virginica* (59% *M. arenaria* vs. 40% *M. edulis*, $P = .001$; 83% *M. arenaria* vs. 22% *C. virginica*, $P = .0001$) indicating a strong preference for soft shell clams. Sexual differences in predation patterns reported here are likely due to differences in the cheliped morphology of male and female crabs.

METHODOLOGY FOR THE GENERATION OF MOLECULAR TAGS IN *PLACOPECTEN MAGELLANICUS* (SEA SCALLOP) AND *ARGOPECTEN IRRADIANS* (BAY SCALLOP). **Maronda V. Brown** and **Linda Strausbaugh**, University of Connecticut, Department of Molecular & Cell Biology, Storrs, CT 06269; **Sheila Stiles**, USDOC, NOAA, National Marine Fisheries Service, Northeast Fisheries Science Center, Milford Laboratory, Milford, CT 06460.

Using various molecular genetic techniques that include restriction fragment length polymorphisms (RFLPs), Randomly Amplified Polymorphic DNAs (RAPDs), and Polymerase Chain Reaction (PCR), we have begun to examine regions associated with molecular markers in the sea scallop, *Placopecten magellanicus*. *P. magellanicus* samples were obtained from several regions off the Northeast Atlantic coast at multiple sites around Georges Bank and from the southeastern Canadian waters. We analyzed samples from both inside & outside of the United States governmental restricted area. Preliminary screening of a *Placopecten magellanicus* genomic library seems to reveal positive clones using primers made to both *Drosophila melanogaster* (fruit fly) histone H2A-H2B and to the *Pisaster ochraceus* (sea star) H3 histone gene. Preliminary comparisons were made with the bay scallop *Argopecten irradians*. Further analyses should result in the identification of a series of polymorphic regions in both *Placopecten magellanicus* and *Argopecten irradians* as well as provide basic molecular biology characterization of the highly conserved histone gene family in mollusks.

EDUCATIONAL PARTNERSHIPS: ITS VALUE TO THE FUTURE OF AQUACULTURE. **John J. Curtis** and **Sherry Lonergan**, Bridgeport Regional Vocational Aquaculture School, 60 St. Stephens Road, Bridgeport, CT 06605.

A simple mathematical equation that has been the driving force of the Bridgeport Regional Vocational Aquaculture School since its inception in the 1988–89 academic year is:

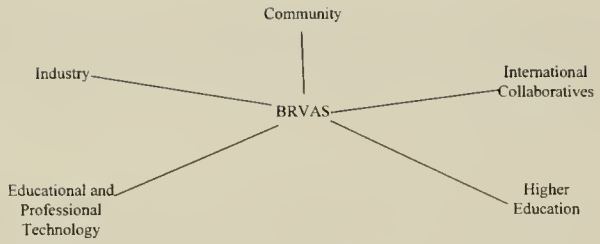

Figure 1. STAR diagram.

Education + Partnerships + Aquaculture = High School Student Success

Education is defined as the development of a person by fostering to varying degrees the growth of expansion of knowledge, wisdom, desirable qualities of mind or character, physical health, or general competence especially by a course of formal study or instruction.

Partnership, by definition, is an alliance of at least two parties with a common interest usually leading to a mutual benefit and accomplishment.

Aquaculture, a rapidly growing global industry whose own success will be dependent on the numbers of young, creative and committed students who we can encourage to pursue it as a lifelong career.

The Bridgeport Regional Vocational Aquaculture School, in its short history, realized from the beginning the value of partnerships. Traditional education will always be the foundation, but like any structure, success is measured by the extent of additions to that foundation. Early on, the administration and staff of the Bridgeport Regional Vocational Aquaculture School committed themselves to not only providing the solid academic foundations but also seeking multiple opportunities for the students to apply their hard-earned knowledge to real-life and meaningful collaborative projects. It worked! And it continues to work! More students are scoring higher on standardized academic tests and there continues to be a significant yearly increase in the numbers of students who pursue related post-secondary education.

Through the dedication of staff and a commitment to introduce students to the value of their own commitment to learning and involvement, they partnered with their students and a STAR (Fig. 1) was born. As a result, the aquaculture industry and the students who choose to attend the Bridgeport Regional Vocational Aquaculture School will continue to benefit from the school's commitment to this simple mathematical equation.

JAMES RIVER MARKET SIZED OYSTERS HAVE THEIR LATE SUMMER SURVIVAL RATES DOUBLED BY MARL TREATMENT OF THEIR WATER. **Russell P. Davis**, Sweet-Water Oysters, 1521 Quail Pt. Rd.,Va. Beach, VA 23454.

James River oysters, market sized at about 250 per bushel, are not normally expected to survive another summer. The cause of death is usually attributed to either MSX or Dermo.

This experiment appears to reproduce conditions under which oysters thrived abnormally well. Prior to this experiment it appears that no one successfully intervened late in the disease process. The closest research is particularly relevant in that iron is proven to be a factor in the Dermo disease process.

A lot of those older oysters were given water that was run through a mesh bag of fossil shell hash. Twice the proportion of oysters survived in the treated water as did in the untreated water.

Approximately, 20.8% of the no-marl oysters survived and 41.7% of the marled oysters survived. Given the sample size and the binomial nature of the survival statistic there is a 1:16 chance that the marl treatment made no difference.

The shell hash was dissolved by the passing water indicating calcium carbonate under-saturation. The existence of instances of calcium carbonate under-saturation appears controversial. The accumulation of iron sulfide in the troughs was surprising and inspired additional inquiry into the geochemistry of iron sulfide. Apparently, extremes in sediment carbonate/sulfide ratios do occur.

When iron sulfide is resuspended and oxidized, the resulting burst of sulfuric acid can produce calcium carbonate undersaturation particularly at the sediment/water boundary layer. This process could explain some disappearance of shell hash and cultch. Additional sediment chemistry experiments shed light upon the geochemical mechanisms behind the abrupt discontinuity in Virginia between prehistoric sediments that are dominantly oxic/carbonate and historic sediments that are dominantly anoxic/sulfide.

These sediment chemistry experiments explored extremes in carbonate/sulfide ratios and suggest that a restoration to prehistoric levels of estuarine productivity is too conservative a goal - Sunlight-limited high goals for shellfish productivity may be easier to achieve than more modest goals provided that "whole-river husbandry" is allowed.

DEVELOPMENTS CULTIVATING BIVALVE MOLLUSCS IN EUROPE. Eric Edwards, Shellfish Association of Great Britain, Fishmongers' Hall, London Bridge, London, EC4R 9EL, UK.

Increasing quantities of bivalve molluscs are now being produced in European waters by various cultivation techniques. European production of bivalves is now around 825,000 metric tonnes a year. Mussels and Pacific oysters represent just over 90% of this total. The lead countries are: France, Spain, Holland and Italy.

Clams are another important species, especially in southern Europe, where a number of different types are cultivated or harvested from wild resources. Exciting developments are also taking place in the culture of scallops, abalone and exotic clams. The 2000s will see increased production of these molluscs using hatcheries and technology adopted from other countries. Historical records show that bivalve molluscs like oysters and mussels have been cultivated in Europe for generations using traditional cultivation methods. But the last 100 years have seen big changes in many of these shellfisheries caused by natural and economic problems.

For example, France was once a major producer of the European flat oyster (*Ostrea edulis*) selling over 500 million a year between 1877 to 1887. But massive mortalities of these native oysters encouraged the French growers to bring in Portuguese cupped oysters. In the 1960's, a viral infection hit the 'Ports', then the parasite *Bonamia* infected the natives and since the 1970s, the French have cultivated the Pacific oyster. As a result, their oyster industry is now dominated by *Crassostrea gigas*, at an annual production level of 150,000 tonnes. Similar changes have also taken place in the UK, Holland and Ireland where oyster production has also dramatically declined.

As a result, mussels have steadily taken over as Europe's premier bivalve species. Once the convenience food of the working classes, mussels have now become a favourite seafood in most European countries, especially France, Belgium and Spain. Two species are grown: the northern blue mussel (*Mytilus edulis*) and the Mediterranean species (*Mytilus galloprovincialis*). Culture methods range from the mechanized bottom system used in the Netherlands to rope culture using rafts or buoys in Spain and Italy. These two methods have also been used successfully, although on a more limited scale, in Ireland, UK, Sweden and Greece.

As well as various natural diseases, Europe's bivalve industry has suffered from the effects of pollution, mainly from coastal sewage discharges and organo-tin (TBT) antifoulant paints used on ship's hull and fish cages. Agricultural run off is also a new problem as are toxic algal blooms. The need to supply safe shellfish has prompted the European Commission to formulate and introduce Shellfish Hygiene Directives which classify harvesting areas and have set bacterial and algal toxin standards for waters and shellfish flesh. The use of approved depuration plants—mainly using ultraviolet light or chlorination to sterilise seawater—has helped to reduce public health outbreaks in many countries. TBT paints, which caused malformations in Pacific oysters and killed bivalve larvae, have since been controlled by national legislation in many European countries.

There is a growing market for bivalve molluscs in Europe and in general the demand is increasing. Exports between Member States are also growing in importance, as more consumers perceive shellfish as attractive, healthy and different. There are opportunities to be had in both niche markets or the bulk market. Added-value products are gaining in popularity, both in terms of improving the produce presentation, convenience and for extending shelf life. Europe's huge bivalve production and the traditional expertise of shellfish producers, along with new technologies, will ensure the continued success of the cultivation industry in the 2000s.

ACQUISITION AND PREVENTION OF *MSX* AND *DERMO* IN A HATCHERY AND LAND-BASED NURSERY: A DNA ASSAY INVESTIGATION. **Susan Ford, Zhe Xu**, and **Gregory DeBrosse**, Haskin Shellfish Research Laboratory, Institute of Marine and Coastal Science, Rutgers University, 6959 Miller Avenue, Port Norris, NJ 08349.

Over the past decade, the two major pathogens of eastern oysters (*Haplosporidium nelsoni* [MSX] and *Perkinsus marinus* [Dermo] have become epizootic in many areas of the northeastern United States where oyster culture depends on hatchery-produced seed. Questions often arise as to whether larval or juvenile stages become infected in the hatchery or nursery phase of production. To help answer these questions, we utilized both traditional and molecular diagnostic methods to detect the pathogens in larval and juvenile oysters reared at our Cape Shore hatchery/nursery on the shore of lower Delaware Bay where both parasites are enzootic. In 1995, we found very low levels of *P. marinus* in juveniles held for 7 weeks in a nursery upweller system receiving raw bay water. Detection was made using the whole-body parasite burden assay and Ray's Fluid Thioglycollate Medium (RFTM). In 1998, we analyzed juveniles in the same upweller system for both parasites. The body burden assay was used for *P. marinus*, whereas *H. nelsoni* was diagnosed with both traditional tissue-section histology and pathogen-specific primer/PCR technology. Oysters resident in the system for 7–8 weeks were infected with both parasites. In 1999, we expanded our investigation to include oysters in the hatchery, as well as in the nursery, and we employed both molecular and classical methods for detection of both parasites. Three spawnings were made at different times during the summer. From each spawning, samples of eyed larvae and 1-mm spat were collected from the hatchery, where water was filtered to 1 μ and UV-treated. Juveniles were taken 5 weeks after deployment in the nursery.

One aliquot of the first spawning remained in the filtered/UV-treated hatchery water for an additional 10 weeks and was compared with animals kept in the raw-water nursery upwellers during the same period. Neither pathogen was detected, using the molecular assays, in eyed larvae, 1-mm spat, or spat held in the hatchery for an additional 5 weeks. A positive response to *H. nelsoni* was detected after 10 weeks in the hatchery, although none was found for *P. marinus*. The PCR assay detected *H. nelsoni* in juveniles held in the nursery, although traditional histology failed to find infections. Unexpectedly, the PCR assay for *P. marinus* failed to find infections in the nursery system, whereas the whole-body RFTM method detected small numbers of parasites. It is clear that oysters in a nursery system receiving raw water pumped from an area enzootic for *P. marinus* and *H. nelsoni* are highly likely to become infected by both parasites, although infections may be very light. Filtration to 1 μ and treatment with UV appear to protect larval and early spat stages from both pathogens, although there was some evidence that juveniles held for a prolonged period in the hatchery did become infected with *H. nelsoni*. Whether this was because of infective particles passing through the system or accidental contamination of the sample at some stage of processing is presently unknown.

THE QUESTION OF TEMPERATURE AND *PERKINSUS MARINUS* (DERMO) ACTIVITY IN THE NORTHEASTERN UNITED STATES. **Susan Ford**, Haskin Shellfish Research Laboratory, Institute of Marine and Coastal Science, Rutgers University, 6959 Miller Avenue, Port Norris, NJ 08349; **Roxanna Smolowitz**, Marine Biological Laboratory, Woods Hole, MA, 02543; and **Marnita Chintala**, Atlantic Ecology Division, U.S. EPA, Narragansett, RI 02882.

During the early 1990s, epizootic outbreaks of Dermo disease, caused by the water-borne parasite *Perkinsus marinus*, occurred over an area from Delaware Bay, NJ to Cape Cod, MA, areas previously free of the disease. The apparent northward movement of *P. marinus* coincided with a pronounced warming trend, particularly during the winter. Knowledge that the parasite is most active at high temperatures led to the hypothesis that the warmer water temperatures had created a more favorable environment for *P. marinus* in the northeastern US, which allowed it to proliferate and spread from localized foci in infected oysters historically introduced from the south. An alternative hypothesis was that a low-temperature tolerant strain of *P. marinus* had developed and invaded northeastern oyster growing areas.

Surprisingly, anecdotal reports from oyster growers indicated that the disease was causing few deleterious effects in the Northeast. It was thought that the lower temperatures and shorter growing seasons might prevent *P. marinus* from reaching harmful levels. To investigate this possibility and to document the relationship between temperature cycles and Dermo disease development in the Northeast, we monitored oyster stocks between Delaware and Cape Cod Bays over a two-year period from 1996 to 1998. We regularly measured *P. marinus* prevalence and intensity; oyster growth, condition index and mortality; and the presence of other disease agents. Results showed that in contrast to the prevailing reports, *P. marinus* behaved in its new range very much as it does in southern areas where it has been enzootic for decades. Seasonal cycles were similar, as was the 2-3 year progression to a full epizootic. The pathogen reduced oyster meat condition, but generally not until oysters had been under disease pressure for two years. Mortality was clearly associated with elevated infection levels, but did not become important until the second year of exposure. Cumulative mortality over the two years of the present study was at least as great as in more southern locations. The most obvious reason for these observations is that temperatures in most of the growing areas examined readily became warm enough to sustain high *P. marinus* proliferation, and winters were not cold enough to limit disease cycles.

The distribution and abundance of *P. marinus* seems to have stabilized after its initial incursion into the Northeast. Its presence has not diminished in areas where it has become well established and it has not intensified in areas that experienced little or no infection pressure during the early 1990s. This second observation does not mean that these areas are likely to remain free of Dermo disease. There are no obvious environmental limits (i.e., temperature or salinity) to the parasite in many of these areas - only a (current) lack of infective elements. There is no reason that epizootics should not occur if more infective particles are introduced into these or adjacent sites, either naturally or through the importation of infected oysters.

Based on an *in vitro* assay of *P. marinus* isolates originating from Massachusetts to South Carolina, there appear to be physiological responses (growth, metabolic activity, or both) to temperature that vary along a latitudinal cline; however, there was no consistent evidence in this assay, or in comparison of *in vivo* proliferation in field samples, that a low-temperature tolerant strain of *P. marinus* was present in the Northeast. Nevertheless, given the current abundance of the parasite in this region, it would be unwise to dismiss the possibility that selection for low-temperature tolerance is underway.

POPULATION ENHANCEMENT EFFORTS FOR THE BAY SCALLOP, *ARGOPECTEN IRRADIANS*, IN THE NIANTIC RIVER ESTUARY, CONNECTICUT, USA. **Ronald Goldberg, Jose Pereira,** and **Paul Clark**, USDOC, NOAA, National Marine Fisheries Service, Northeast Fisheries Science Center, Milford Laboratory, Milford, CT 06460; **Bernard Faber, David Porter, Lorenz Rinek, Barbara Kanter, Eric Kanter,** and **Walter Lord,** Waterford East- Lyme Shellfish Commission, 5 Rope Ferry Road, Waterford CT 06385.

The Niantic River supports presently a small population of bay scallops, *Argopecten irradians*, that is harvested recreationally. Numbers of bay scallops have fluctuated greatly with a peak level estimated as high as 20 million animals in the 1940s (Marshall, 1960). Three potential enhancement strategies were evaluated: 1) collection of natural spatfall, 2) direct re-seeding, and 3) overwintering of hatchery-reared stock for creation of spawner sanctuaries. Assessment of natural spatfall in 1997 indicated that peak spawning occurred in late July and that spat were dispersed widely, however, too few spat were available for enhancement activities. In direct re-seeding experiments, time of planting and the inferred predation intensity were major factors affecting survival, while planting density had no significant effect. The Waterford East-Lyme Shellfish Commission (WELSCO) held 26,000 bay scallops in suspension culture during the 1998 – 1999 winter, of which, 60 – 80 % survived and spawned during the summer of 1999 within mobile spawner sanctuaries (cages suspended on long-lines). This effort is being repeated during 1999 – 2000. An annual recreational harvest survey has been initiated to assess enhancement activities. The pro-active approach of WELSCO in using aquacultural methods for enhancement of bay scallop populations is appropriate when natural recruitment is poor and habitat and environmental conditions are not limiting.

BREEDING FOR A SUPERIOR EASTERN OYSTER FOR THE NORTHEASTERN REGION. **Ximing Guo, Susan Ford,** and **Gregory DeBrosse**, Haskin Shellfish Research Laboratory, Institute of Marine and Coastal Science, Rutgers University, 6959 Miller Avenue, Port Norris, NJ 08349; **Roxanna Smolowitz**, Marine Biological Laboratory, 7 MBL Street, Woods Hole, MA 02543.

The eastern oyster, *Crassostrea virginica*, supports a major aquaculture industry in the Northeastern (NE) region. An important need of this industry is disease-resistant and faster growing stocks. The eastern oyster faces three major diseases in the NE region: Dermo (caused by the parasite *Perkinsus marinus*), MSX (caused by the parasite *Haplosporidium nelsoni*) and JOD (causative agent unknown). Rutgers University has maintained an oyster breeding program since the early 1960s, established by Harold Haskin. Rutgers resistant strains have shown strong resistance to MSX and recently some resistance to Dermo. They have not been exposed to JOD and are probably susceptible to this important disease. Rutgers strains have recently been re-grouped into a mid-Atlantic strain (DBH) and a Northeastern strain (NEH). The NEH strains are originated from the NE region and known for their better growth compared to the DBH strains. Research funded by Sea Grant and the State of New Jersey is underway to enhance further the disease resistance and growth of the NEH strain.

To obtain JOD-resistance, the NEH strain will be crossed with a JOD-resistant strain from the FM Flower Oyster Company (NY) and evaluated for resistance throughout the NE region. To enhance growth significantly, we plan to develop a tetraploid stock of disease-resistant strains for the production of all-triploid and disease-resistant oysters. Triploids are organisms with three sets of chromosomes instead of two sets found in normal diploids. Triploid shellfish grow significantly faster than normal diploids in almost all shellfish studied so far. Triploid eastern oysters produced by chemical induction grow 12-41% faster than diploids, and triploids produced from tetraploids are expected to grow even faster. The enhanced growth reduces not only production duration, but also exposure to diseases. Triploid oysters also have improved meat quality during the summer season. The combination of disease-resistant strains and the triploid-tetraploid technology should produce a superior stock for the NE oyster industry.

STATE IMPLEMENTATION OF THE NSSP INTERIM CONTROL PLAN FOR *VIBRIO PARAHAEMOLYTICUS*. **J. Michael Hickey**, Interstate Shellfish Sanitation Conference, 115 Atrium Way, Suite 117, Columbia, SC 29223.

Vibrio parahaemolyticus (Vp) is a bacterium found commonly and at seasonally high numbers in coastal waters worldwide. Some serotypes of this organism cause gastroenteritis in the general population, not solely in high-risk groups, as is the case for *V. vulnificus*. Sporadic cases along with occasional outbreaks of gastroenteritis have been linked to consumption of raw seafood, including molluscan shellfish, and seafood re-contaminated after cooking. The actual incidence of these illnesses is unknown since, like many foodborne illnesses, this disease is self-limiting and those affected may not seek treatment. Also, states vary considerably in their *Vp* investigations and reporting practices. However, four outbreaks in 1997 and 1998 resulting in over 700 cases have been traced to consumption of raw oysters and some additional seafood products from Texas, New York, and the Pacific Northwest. These issues and the growing evidence of more virulent strains of *Vp* involved in many of the cases have initiated a concerted effort by the shellfish industry, state and federal regulators through the ISSC to better understand the organism and limit risk to shellfish consumers.

The Interstate Shellfish Sanitation Conference (ISSC), in 1998, adopted an interim control plan for addressing illnesses associated with *Vibrio parahaemolyticus*. This interim control plan was modified by the ISSC in 1999 to incorporate the use of a gene-probe for identification of pathogenic strains of *Vp*. The presentation will provide an update of recent ISSC actions and include a report regarding state implementation of the interim control plan for *Vp*.

THE ECONOMICS OF BLUE MUSSEL GROW-OUT: AQUACULTURE AT AN OFFSHORE SITE. **Porter Hoagland** and **Hauke L. Kite-Powell**, Marine Policy Center, Woods Hole Oceanographic Institution, Woods Hole, MA, 02543.

It is widely believed that the commercialization of ocean mariculture is an area of tremendous future economic potential. In the United States, this perceived potential exists because of an increasing longterm trend in the per capita consumption of seafood, limits on the output of some important commercial wild harvest stocks, the availability of offshore locations that both minimize the possibility of conflicts with other uses and permit operations large enough to achieve efficient scale economies, and good water quality, among other reasons. However, several serious scientific questions, technological problems, and economic and policy issues must be clarified or resolved before this potential can be realized. As a problem of economic development with the potential to benefit coastal communities, the central issue is one of reducing risks arising from all of these areas to levels that improve the likelihood of investment flows.

With the assistance of Blue-Gold Ltd., located in New Bedford and the largest U.S. mussel processor, scientists and engineers at WHOI are taking an interdisciplinary approach to the development of a framework for reducing the risks of ocean mariculture operations, focusing on the production of the blue mussel (*Mytilus edulis*). Suspended ocean culture of mussels is likely to result in a product of superior taste and quality, free of pearls and grit contamination, and with reduced levels of commensal organisms and predation. We are combining offshore engineering, biological studies, and the analysis of project economics to characterize a technically optimized and commercially feasible submerged suspension structure. Here, we report on the continuing development of a framework for evaluating the commercial viability of offshore farming.

We define a full scale offshore mussel farming operation to be one that fully utilizes the annual capacity of one service vessel (*e.g.*, a small scalloper of approximately 20 GRT). Such a vessel requires fixed cost payments on the order of $80,000 per year. Daily variable costs (fuel and supplies) are $1400, including $800 in crew member wages. We estimate that one vessel is capable of servicing a field of 300 longlines. We assume that 150 longlines are harvested once every two years. Each year, about 225 days are spent maintaining the longlines and 38 days are required for harvesting. During years when the longlines are being deployed, an additional 38 days are required to deploy one-half the field (150 longlines). Larger farms can be scaled as multiples of this basic farm.

Each longline is designed to support 25 mussel socks or ropes. Each sock produces, on average, 350 pounds of mussels over a two-year growout period. A longline costs $400 to deploy and $250 to maintain each year, not including the costs of running the service vessel. Each longline is designed to last ten years, at which time it must be replaced. Additional costs include those associated with processing (sorting, debearding, and cleaning), transport to the market, and management costs.

Prospective offshore aquaculture entrepreneurs face a wide variety of environmental, engineering, economic, and regulatory risks. Many specific risk categories cannot be characterized fully until offshore aquaculture takes place on a commercial scale. Nevertheless, our model has been developed to handle two main types of risk: market and production. These risks are simulated using assumed distributions for the relevant parameters: price and natural growth.

A model of market price is estimated from the monthly distribution of the value per pound of imported Canadian cultured blue mussels during 1990-97 (averaging 91¢ per pound). We assume that price is distributed lognormally with a mean of 64¢ per pound and a standard deviation of 11¢ per pound. The mean has been adjusted downward to account for the effect of domestic production on market price.

Developing a model of production risk is more problematic in the absence of a history of offshore production activity. This parameter can be affected by storm events, predation, parasitism, disease, temperature, and availability of food, among other things. We assume that production takes an extreme value distribution with a mode of 380 pounds per sock and a scale of 50 pounds. The majority of possible values for production from a sock thus range between 80 and 480 pounds per sock.

We assume a two percent rate of inflation, and we discount revenues and costs at a rate of 12 percent. The model predicts a marginally profitable operation with a discounted cash flow of $0.3 million over a thirteen year period. This result suggests that full scale mussel farming at an offshore location is commercially feasible. However, the possibility of losses is not insignificant. Farm profitability is greatly enhanced if the current market price for Canadian aquaculture product is used in the model. We expect to refine the model as we gain more experience with the prototype longline.

This research has been sponsored with funds from the U.S. Department of Commerce, NOAA, National Sea Grant College Program under grant Number NA86RG0075 (Woods Hole Oceanographic Institution project number R/A-40) and the WHOI Marine Policy Center.

SHELLFISH PRODUCTION IN THE BLAKE FLOATING HATCHERY AND IN MODIFIED TIDAL UPWELLER NURSERIES IN 1999. **Richard C. Karney**, Martha's Vineyard Shellfish Group, Inc., Box 1552, Oak Bluffs, MA 02557; **John C. Blake**, Sweet Neck Farm, Box 1468, Edgartown, MA 02539; and **Thomas E. Berry**, Martha's Vineyard Shellfish, Box 1660, Edgartown, MA 02539.

With funding from the National Fish and Wildlife Foundation and the Massachusetts Department of Food and Agriculture two innovative field shellfish culture systems were successfully demonstrated. The floating hatchery/nursery and the modified tidal upweller nursery were both designed by Edgartown aquaculturist, Jack Blake.

First tested in 1998, the Blake Floating Hatchery/nursery prototype was modified in 1999 with the addition of a 400 watt wind turbine which reduced the labor of recharging and exchanging the four 6 volt batteries which powered the system. The prototype was tested in three culture modes: as a 340 gallon larval tank, as a nursery capable of holding eight downweller sieves for post set culture, and as a nursery with eight upweller silos for rearing juveniles. All except the flow through larval culture mode proved successful.

In the first culture trial, 20 million quahog embryos were introduced into the larval tank which received an approximate flow of 146 gallons of 5 micron bag-filtered seawater per hour. The tank was fitted with three 51 micron exit sieves with a surface area of about 700 sq. inches to prevent loss of larvae. By Day 3, the tank was contaminated with copepods and the larvae were observed to be hollow looking and in poor health. The poor condition of the larvae resulted from either a lack of food due to competition from the copepods or stress related to impingement on the mesh of the exit sieves. The copepods were thought to have entered the system when waves pounded the exit sieves.

In the second culture trial, 10 million 48 hour old oyster larvae were successfully taken to the eyed stage in a closed, aerated, larval culture with a daily addition of cultured phytoplankton and a change of seawater every second day. Approximately 1.6 million eyed larvae were set on crushed oyster and poultry shell cultch in eight downweller sieves. The post set were cultured in the system's upweller silos which received a maximum flow of about 10 thousand gallons/silo/day and resulted in the production of 130,000 single seed oysters.

Blake's modified tidal upweller was designed to maximize the flow of water through the unit by eliminating flow constricting outlet ports. In place of standard upweller silos, Blake's upweller uses stacked bins to hold the seed. The nursery has 24 support racks, each of which can hold three stacked bins. On 6 August, 1999 during a time of average tides, the flow rate through the tidal upweller measured with a Marsh McBirney Paramagnetic Current Sensor was determined to be about 175 gallons per minute.

Over the course of the 1999 growing season, over 0.5 million oyster seed and 1.4 million quahog seed were cultured in four upweller nurseries. The oyster seed (3–7 mm) was set out in batches throughout the growing season, between 21 June and 8 September. On average, the oyster seed were rinsed clean about twice a week, sieved to size every week or two, and thinned in half about every ten days. At the beginning of the culture, the seed was about 4–5 mm in size, and held at an initial density of about 20,000 per bin. Within about six weeks, the oysters had grown to about 37 mm and were at a density of about 1,400 per bin. When they reached about 37 mm the oysters were transferred from the upweller nurseries into growout cages. Oyster seed survival in the units was nearly 100%.

Quahog seed, at about 0.75 mm, was introduced into two of the upwellers on 25 June and 3 July at an initial density of about 180,000 per bin. It was rinsed clean about every two to five days (more frequently when it was smaller). After about three weeks it was thinned to a density of about 60,000 per bin. After about a month, it grew to an average size of about 5.7 mm. Quahog seed mortality was reported to be about 5–10%.

An experiment was conducted to determine if bins built from ACQ™ pressure treated lumber would have any negative impacts on the growth of small seed. Equal numbers of 2-3 mm oyster seed were placed into upweller bins, one with a pressure treated frame, and the other with a frame of untreated painted wood. After two weeks, a sample of seed from each treatment was measured and the difference in growth was found to be insignificant.

OPERATIONS AT TAYLOR SEAFOOD. Gordon King, Taylor Resources Inc., Shelton, WA 98584.

Taylor United, Inc. has been growing *Mytilus galloprovincialis* for approximately ten years. Presently we market about 800,000 pounds per year. All production is hatchery seed based and grown out on rafts with average spawn to harvest of 16–18 months. The company also produces about 140,000 gallons of shucked oysters, one-million dozen single oysters and 3,000,000 pounds of manila clams. Recently, Taylor Seafood has been planting two million geoduck seed a year and should start harvesting significant volumes in the next two years. The company has a hatchery in Quilcene, Washington, a floating upwell nursery in Shelton, Washington, a leased hatchery in Tillamook, Oregon and a hatchery nursery in Kona, Hawaii. We also recently started a scallop farm in Mexico.

These operations will be described in the presentation.

SUBMERGED LONGLINE CULTURE OF BLUE MUSSELS (*MYTILUS EDULIS*) AT AN OPEN OCEAN SITE IN THE GULF OF MAINE. Richard Langan, Jackson Estuarine Laboratory, University of New Hampshire, 85 Adams Point Road, Durham, NH 03824.

In the northeastern US, competing and often conflicting uses limit the availability of protected inshore sites for shellfish culture. Though not totally without conflict, open ocean sites provide greater opportunity for shellfish culture. However, the greater depth and rigorous conditions in the open sea require engineering adaptations of the existing technology. The University of New Hampshire, in collaboration with the Portsmouth, NH Commercial Fishermen's Cooperative, has established a pilot-scale submerged longline culture system in order to demonstrate the feasibility of culturing mussels in the open ocean environment. The project is located at a fully exposed site eight kilometers from shore in the open waters of the Gulf of Maine.

In the spring of 1999, a longline system consisting of large (3200 kg) concrete anchors spaced 180m apart, 30 mm polysteel line for anchor and headlines, and both steel and hard plastic submersible buoys for floatation was deployed. The depth at the site is 52 m, and the horizontal headline is submerged 15 m below the surface. A standard 12.5 m lobster fishing boat was outfitted with deck gear needed to tend submerged longlines. In June 1999, one hundred 12 m mesh socks filled with mussel seed collected in the summer of 1998 were deployed vertically from the headline. In October 1999, seed collected in spring of 1999 were used to fill mesh socks 60 m in length that were attached to the longline in a looping pattern. Mussel growth and density, and physical and biological conditions at the site have been monitored monthly.

Mussel growth for the 1998 year-class of seed has averaged 1.6 mm per week for the period June 1999 to November 1999. This rate of growth suggests that a period of 15 to 18 months post-set would be required for mussels to reach market size. The longline has thus far been unaffected by severe weather and wave heights in excess of 6 m. Preliminary economic analysis indicates a favorable outlook for both small and large-scale operations.

DETECTING THE PRESENCE OF *PERKINSUS MARINUS* AND *HAPLOSPORIDIUM NELSONI* IN THE OYSTER, *CRASSOSTREA VIRGINICA*, IN RHODE ISLAND WATERS: A SURVEY UPDATE. Ken Leonard III, Josefa Dougal, and **Marta Gomez-Chiarri**, Fisheries, Animal and Veterinary Science, University of Rhode Island, Kingston, RI 02881; **Arthur Ganz**, Rhode Island Department of Environmental Management, Coastal Fisheries Laboratory, Wakefield, RI 02879.

Dermo and MSX, diseases caused by the parasites *Perkinsus marinus* and *Haplosporidium nelsoni* respectively, have been responsible for oyster mortalities throughout the east coast of the United States. We report here the latest results from an oyster disease survey that began in May 1998. Oysters (30 per site) were collected from 8–10 locations in Rhode Island, including 2 aquaculture sites, in May, August, and November 1998, and February, August, and November 1999. The prevalence and intensity of Dermo infections were evaluated using the Ray's Fluid Thioglycollate Medium (RFTM) method. MSX infections were evaluated using histological examination. *Perkinsus marinus* and *Haplosporidium nelsoni* infections were detected in August 1998 and continued to be present in samples collected in August and November 1999. The highest weighted prevalences of *Perkinsus marinus* were observed in August 1998 and 1999 in samples from Barrington River, Wickford Cove, and Charlestown Pond, three locations historically known to support oyster populations. Oyster mortalities reported at these locations may be due to high *Perkinsus marinus* infections. Some initial and intermediate *Haplosporidium nelsoni* infections were observed in oysters from 5 locations. No advanced infections have been detected so far.

FEDERAL CROP INSURANCE BECOMES AVAILABLE FOR QUAHOG FARMERS. Dale F. Leavitt and **William Burt**, SouthEastern Massachusetts Aquaculture Center, Buzzards Bay, MA 02532; **Charles Koines**, Risk Management Agency – USDA, Ballston Spa, NY 12019.

What do quahogs have in common with soy beans, cranberries, and corn? They are all agricultural crops that are recognized by the federal government for inclusion in a federally subsidized crop insurance program. In a pilot program developed by the United States Department of Agriculture (USDA) Risk Management Office, quahog farmers in areas within Massachusetts, Virginia, South Carolina, and Florida are eligible for crop insurance. The 1999 crop insurance program was initiated in November 1999 with a cut-off date of 1 December for this year's crop. At this point, the

crop insurance program covers the grow-out phase of hard clam culture and the field planted seed quahogs have to be larger than 10 mm to be eligible for insurance. The crop insurance program will be further developed during the next three years to include the nursery stage of clam farming and possibly clam hatcheries. An overview of the quahog crop insurance program will be presented with insight as to how it was developed by the USDA using field agents and the industry to set the standards.

PRODUCTION OF TRANSGENIC MOLLUSKS AND CRUSTACEANS. **Chun-Mean Lin, Sineenat Siri,** University of Connecticut Biotechnology Center, Storrs, CT 06269; **Sheila Stiles**, USDOC, NOAA, National Marine Fisheries Service, Northeast Fisheries Science Center, Milford Laboratory, Milford, CT 06460; and **Thomas Chen,** University of Connecticut Biotechnology Center, Storrs, CT 06269.

It has been demonstrated that introduction of naked DNA into newly fertilized or unfertilized eggs by microinjection or electroporation reproducibly results in production of transgenic finfish. However, almost no examples have been shown concerning production of transgenic mollusks and crustaceans by direct microinjection and electroporation of naked DNA into these animals. Recently, a new gene transfer vector, defective pantropic retroviral vector, has been developed that contains a glycoprotein (VSV-G protein) from Vesicular Stomatitis Virus (VSV). We have demonstrated successful transfer of foreign genes into dwarf surfclams or medaka by electroporating the newly fertilized eggs or exposing the developing gonads to these gene transfer vectors. We, therefore, believe that the same gene transfer technology may be used to produce transgenic crustaceans.

Defective pantropic retroviral vector containing a lacZ or neoR reporter gene was introduced into immature gonads of male and female crayfish immediately post the last larval molt by microsurgery. The treated animals were maintained in separate aquariums with respective untreated females or males for maturation and spawning, and the newly hatched larvae were collected for rearing. Leg appendages from the presumptive transgenic animals were removed for the determination of the presence of the lacZ or neoR transgene by PCR amplification. Results of PCR amplification analysis showed that ranging from 30 to 50% of the progeny produced by the treated males or females contained the lacZ or neoR transgene. Integration of the transgene into the host genome was further confirmed by linker mediated PCR of the genomic DNA isolated from the PCR positive animals. Expression of the transgenes in the transgenic animals was also detected by the reverse transcription/PCR analysis. These results demonstrate conclusively that crustaceans can be produced routinely by infecting the immature gonads with defective pantropic retroviral vectors carrying desirable transgenes.

Recently, we have also introduced a common carp B-actin promoter trout GH cDNA transgene into newly fertilized bay scallop eggs by electroporation. A significant fraction of the hatched animals are shown to carry the GH transgene and have grown to adulthood. F_1 transgenic animals have also been produced by crossing P_1 transgenics with non-transgenics. These results showed that transgenic bay scallops can be produced by electroporating naked DNA into newly fertilized embryos and the transgene can be transmitted into the subsequent generation. Work is underway to characterize the integration and expression of the transgene and the performance of the transgenic progeny. (This research is supported by a grant from the Connecticut Sea Grant Program to Thomas Chen).

A REVIEW OF CURRENT CLAM CULTURE IN NEW ENGLAND. **Clyde L. MacKenzie, Jr.,** USDOC, NOAA, National Marine Fisheries Service, Northeast Fisheries Science Center, James J. Howard Marine Sciences Laboratory, Highlands, NJ 07732.

This review includes the culture of softshell clams, *Mya arenaria*, in Maine, and northern quahogs, *Mercenaria mercenaria*, in Massachusetts, Rhode Island, and Connecticut. Clam and quahog culture practices differ among the four states.

Clam culture in Maine consists of spreading hatchery seed and natural seed on the intertidal public clam beds in several towns in northeastern Maine. The hatchery seed is produced in the state's only clam hatchery at Beals Island, while the natural seed is dug in the high areas of flats where it would die if left in place and then transplanted to low areas in the same flats. The stimulus for constructing the hatchery was light clam sets in Washington and Lincoln Counties for approximately 20 years. During the past few years, however, natural setting has increased which may cause the need for planting hatchery seed on the public beds to diminish.

In Massachusetts, quahog hatchery-growout culture occurs on public beds and private leases and has been increasing during the 1990's. At least 20 towns purchase quahog seed to spread on their beds for public fishermen to harvest after they attain market size, and about 250 leaseholders in various towns grow quahogs under plastic screens to avoid predators; leases range from one to ten acres in size. Compared with wild quahog, hatchery quahogs (notata strain) have relatively brittle shells and gape more in storage. The seed is purchased from hatcheries in Maine, Massachusetts, and New Jersey. The state also transplants quahogs from uncertified waters to public town waters.

Culture in Rhode Island consists of a state-run program of transplanting quahogs from uncertified grounds to certified grounds and then holding them for as long as a year until they depurate and also spawn. Only a few acres of bottom are leased for shellfish culture.

In Connecticut, all quahog harvesting takes place on private leases. Quahog culture consists of transplanting stocks from remote beds to beds closer to ports. This enables boats to harvest quahogs more easily during adverse weather. Quahog abundance has increased sharply in Connecticut during the 1990's. MacKenzie and Pikanowski (1998) believe the cause of this has been diminished numbers of starfish, a quahog predator, during that decade. The practice of hatchery-growout culture of quahogs would be difficult because few intertidal areas are available for leasing and most beds are too deep, 3–12 m, to grow quahog seed under screens for predator control.

SYMBIOTIC RELATIONSHIP OF THE SEA SCALLOP AND RED HAKE AS A POSSIBLE MANAGEMENT TOOL. **Mark S. Miller** and **Joseph K. Buttner**, Northeastern Massachusetts Aquaculture Center and Department of Biology, Salem State College, Salem, MA 01970.

The symbiotic relationship between the sea scallop (*Placopecten magellanicus*) and red hake (*Urophycis chuss*) in near coastal waters of New England was monitored by researchers using scuba gear. Scallop beds off the coast of Salem, MA were sampled on a monthly basis as conditions permitted between February and December 1999. After being brought to the surface, scallops were measured and opened to extract hake. Collected hake were counted, measured, and transported to the Cat Cove Marine Laboratory, Salem State College.

Water depth ranged from 18 to 21 m, and bottom temperature varied between 2° and 15 °C. All dives were conducted adjacent to the mouth of Salem Sound along a line running roughly north and south between the coordinates of 42°32.857′ N × 070°48.757′W and 42°30.502′N × 070°47.032′W. A total of 2,978 scallops and 1,421 hake were collected or 0.48 hake per scallop. Percent of scallops with hake varied seasonally, declining sharply to 0% in July and increasing to 96% in mid September as large numbers of young-of-the-year appeared. Scallop size remained fairly constant (avg. = 119.4 mm, S.D. = 14.3 mm). Fish length ranged from 44 to 122 mm, reflecting recruitment and growth. Individual scallops frequently contained two to four fish; the greatest incidence occurred in October, when multiples represented 29% of fish collected. Fish frequently exited scallops after collection, and 22% of all hake were collected as free fish.

Hake transported alive to the laboratory were transferred to 1900 L recirculating systems and readily ingested commercial feed within 24h. Fish fed, survived, and grew despite problems associated with the newly set up facility. On occasion, water temperature rose above 25 °C and total ammonia nitrogen exceeded 15 ppm. Average weight of fish increased from 3.4 g on 12 April to 320 g on 2 December 1999.

Collected data indicate that significant numbers of red hake are lost when scallops are harvested, a potential bycatch of one fish for every two scallops. Our data suggest that scallop harvest conducted between June and August would minimize the coincidental catch of hake. Alternatively, if hake were collected and retained during the colder part of the year (October to March) when post capture mortality was minimal (<30%) and fish were most abundant, the potential exists for commercial culture or stock enhancement.

THE CULTURE OF HADDOCK, *MELANOGRAMMUS AEGLEFINUS*, USING A RECIRCULATING SYSTEM IN AN URBAN SETTING. **Brandy M. Moran, Clifford A. Goudey,** and **Jessica Rabe**, Massachusetts Institute of Technology, Sea Grant College Program, MIT Bldg. E38 - 300, 292 Main Street, Cambridge, MA 02139.

The techniques of recirculating aquaculture are applicable to an urban setting because of the characteristically high growing densities and low water usage of aquaculture facilities. In addition, many coastal urban centers have lost their maritime industrial base and are seeking commercial activity that can maintain the vitality of their waterfronts. MIT Sea Grant College Program, recognizing the potential opportunities that exist for urban aquaculture, has begun a program of research and outreach aimed at promoting sustainable economic development of Boston Harbor based on marine finfish culture. Our Marine Finfish hatchery was dedicated in May 1998. We are now expanding our facilities to increase our production potential and explore additional species as candidates for commercial exploitation.

We have developed techniques for culturing haddock, *Melanogrammus aeglefinus*, in a pilot commercial-scale recirculating system at the hatchery. Light intensity is maintained at 1500 lux. Eggs are incubated in an up-welling concept and held at a constant temperature of 8 °C (+/– 1). Enriched rotifers and *Artemia* are initially offered to the larvae at the density of 7 animals/ml on D2 and D15, respectively. No natural feed supplements or algae are used in the culture methods. Culture temperatures are increased by 1.5 °C over a week each time there is a change to a new feed type. A dry commercial weaning diet is offered to the larvae on D30. The amount of live feed offered to the larvae is decreased until they are completely weaned onto the dry diet. Grading continuously occurs during the juvenile stage.

A haddock growth model has been developed and will be tested against the actual culture of the fish during this 2000 season. The model was used to determine the tank sizes for the facility by

predicting growth rates, survivorship and acceptable densities within a recirculating system. Fish from this year's spawn will be reared for growth trials in the Isles of Shoals Open-Ocean Aquaculture test cages starting in May 2001. A control population will be maintained by MIT to compare the ocean trials with recirculating tank culture at optimal temperatures.

GROWTH OF JUVENILE TAUTOG FED COMMERCIAL DIETS IN A CROSS-OVER EXPERIMENT. **Dean M. Perry,** USDOC, NOAA, National Marine Fisheries Service, Northeast Fisheries Science Center, Milford Laboratory, Milford, CT 06460; **Laurel Ramseyer,** Wildlife Conservation Society, Bronx, NY 10460; **Joseph Goncalo**, 231 Harrison Ave., Milford, CT 06460.

A 2×2 cross-over experimental design was used to evaluate the growth of tautog, *Tautoga onitis*, fed two commercial feeds and reared in twin recirculating systems. An additional objective was to determine whether a simple 2×2 cross-over experimental design was appropriate for a short-term feeding trial. Seventy juvenile ($\bar{x} = 13$ g) tautog were assigned randomly to tanks across systems ($n = 3$). Tautog were fed either Zeigler High Performance Trout Grower or BioKyowa C for 28 d, then switched to the alternate diet for the following 28 d. Diets were fed at a rate of 3% wet body weight/day (dry matter basis). Daily rations were divided into 4 meals/day, and fed with automatic feeders. Tautog fed BioKyowa had significantly higher weight gain (0.50 g/d, $P < 0.001$), higher thermal growth coefficient (11.8×10^{-4}), and lower feed conversion ratios (0.97) than fish fed Zeigler (0.28 g/d, 7.58×10^{-4}, and 1.5, respectively) during the first 28 d of the experiment. Unbalanced carry-over effects resulting from switching the diets at day 28 precluded full use of the 2×2 cross-over model. A switchback design or a 2×2 design with a wash-out period may be more appropriate if unbalanced carry-over effects are expected. Since water quality was not significantly different in tanks of fish assigned to the two different feeding regimes, simple randomization of treatments across recirculating systems would have been sufficient to control for system effects in this experiment. However, maintenance of comparable conditions in two separate systems can never be guaranteed, so further development of cross-over designs is warranted.

GROWING ROTIFERS ON SINGLE AND MIXED ALGAL STRAINS TO BE USED AS A FIRST FEED FOR LARVAL TAUTOG. **Dean M. Perry** and **David A. Nelson,** USDOC, NOAA, National Marine Fisheries Service, Northeast Fisheries Science Center, Milford Laboratory, Milford, CT 06460; **Joseph E. Goncalo**, 231 Harrison Ave., Milford, CT 06460.

This research evaluated the population growth of rotifers (*Brachionus plicatilis*) fed four individual unicellular algal strains; *Dunaliella tertiolecta* (DE), *Nannochloropsis* sp. (UTEX 2341), *Isochrysis* sp. (T-Iso), and *Tetraselmis chui* (Ply 429), or a mixture or "cocktail" of three algal strains; DE, T-Iso, and Ply 429. An additional objective was to determine whether certain algal strains known to contain high levels of the n-3 and n-6 series of polyunsaturated fatty acids used for enrichment of rotifers, also promote high densities of rotifers. The algal cells of the four individual strains used to feed the rotifers averaged 6×10^6 cells/ml. Counts of rotifers grown on these single algal strains were done once a week for 12 weeks. Rotifer populations fed DE, UTEX 2341, T-Iso, or Ply 429 increased an average of 29.8X, 18.5X, 19.1X, and 28.7X, respectively. The algal cells of the three mixed strains used to feed the rotifers averaged 5.0, 6.0, and 30.0×10^6 cells/ml for DE, Ply 429, and T-Iso, respectively. Counts of rotifers grown on a "cocktail" of these three algal strains were done at 4d and at 7d over a 9-week period. Rotifer populations fed the algal "cocktail" increased an average 3.8X at 4d, and 5.4X at 7d. Results of rotifers fed single strains of algae indicate DE and Ply 429 to be the best choices to feed rotifers to maximize reproduction. *Dunaliella*, while promoting good reproduction, is lacking in essential fatty acids. *Tetraselmis* (Ply 429) is a better algal strain to feed rotifers, since it contains the n-3 and n-6 polyunsaturated fatty acids that are necessary for larval marine fish growth and survival. The algal "cocktail" increased the rotifer population dramatically within the first 4d, showing continued reproduction to the seventh day. Although feeding rotifers single algal strains appears to promote higher rotifer densities than feeding them the mixed algal strains, initial rotifer stocking density and culture container size can both be limiting factors that affect population size.

STATUS REPORT FOR THE CHARACTERIZATION OF THE BAY SCALLOP, *ARGOPECTEN IRRADIANS*, GENOME. **Enrico Picozza** and **Joseph Crivello**, University of Connecticut, Department of Physiology & Neurobiology, Storrs, CT 06269; **Maronda V. Brown** and **Linda Strausbaugh**, University of Connecticut Department of Molecular & Cell Biology, Storrs, Connecticut 06269; **Sheila Stiles**, USDOC, NOAA, National Marine Fisheries Service, Northeast Fisheries Science Center, Milford Laboratory, Milford, CT 06460.

The bay scallop (*Argopecten irradians*) is a valuable food source as well as an important inhabitant of marine estuaries and ecosystems, yet little is known about its genome. As we are learning with other organisms, it is important to have a better understanding of the genome structure, and more specifically the structure of particular genes. This will allow us in the future to select scallops for various properties such as increased growth or surviv-

ability for aquaculture, or even as environmental indicators of their micro-environments.

In an attempt to begin understanding the genome of the bay scallop, we spent time developing various tools critical to this task. A genomic library was generated to serve as the basic tool for breaking down the genome into a manageable size. A cDNA library was also generated that will allow us to examine and understand expressed genes and to generate an Expressed Sequence Tags (EST) database. The EST database can be used for managing the functional components of the genome.

Finally in an attempt to begin applying these tools, we are in the process of cloning the metallothionein (mt) gene, a gene that has been shown in other organisms to serve as an indicator of animal exposure to various hazardous pollutants. Details will be presented of the various tools and of the progress being made in the cloning of the mt gene.

OBSERVATIONS ON MYCOBACTERIOSIS IN THE TAUTOG (*TAUTOGA ONITIS*). **Steven Pitchford** and **Richard Robohm**, USDOC , NOAA, National Marine Fisheries Service, Northeast Fisheries Science Center, Milford Laboratory, Milford, CT 06460; **Sharon MacLean**, USDOC , NOAA, National Marine Fisheries Service, Northeast Fisheries Science Center, Narragansett Laboratory, Narragansett, RI 02882; **Laurel Ramseyer**, Wildlife Conservation Society, Bronx, NY 10460.

During the spring and summer of 1999, mortalities occurred in 1-2 year old juvenile tautog being held for nutritional studies at the Milford Laboratory. Internal examination of the dead fish revealed that most contained large numbers of white, round nodules. Nodule imprints and histological sections stained with Kinyoun Acid-Fast stain revealed the presence of acid-fast, rod-shaped bacteria. To prevent infection of other tautog at the facility and to determine the extent of infection, the remaining fish were sacrificed and examined for the presence of nodules. Thirty six of the 123 fish examined (29%) had evidence of nodular growth.

Multiple organ involvement of nodule growth was found in 71.43% of the infected fish. Nodules were found primarily in kidney tissue (85.71%), liver (57.14%) , and spleen (45.71%). Nodules also were seen to a lesser extent in the heart (20.0%), visceral membrane (11.4%) and one instance each in the intestines and gonads (2.9%). With the exception of a few heavily infected fish which became lethargic and had swollen abdomens, very few signs of external pathology were noted.

Slow-growing, acid-fast bacteria were isolated from the nodules using Middlebrook 7H10 Agar media supplemented with ADC enrichment media. After 2–3 weeks, visible growth of raised, roughly textured, off-white colonies was observed. Ongoing and future plans for these isolates call for identification to species level and pathogenicity testing by challenge in healthy tautog.

Several species of *Mycobacteria* have been found to cause diseases in a number of aquatic species including frogs, amphibians, and both freshwater and saltwater fishes. Some of the these same organisms also have been known to infect humans. This is the second known occurrence of mycobacteriosis in the tautog or the wrasse (*Labridae*) family of fishes. Aquaculturalists should be made aware of the possible infection of their stocks by this pathogen.

THE PRESENCE OF *VIBRIO PARAHAEMOLYTICUS* IN *CRASSOSTREA VIRGINICA* AT SPECIFIC LOCATIONS ALONG THE CONNECTICUT AND LONG ISLAND SHORE - PARTICIPATION IN THE *VIBRIO PARAHAEMOLYTICUS* ISSC - FDA SURVEY FOR JUNE 1999 TO JUNE 2000. **Leonora Porter** and **Eugene Zamojcin**, State of New York, Department of Environmental Conservation, 205 North Belle Mead Rd., East Setaucket, NY 11733; **Joe DeCrescenzo, Inke Sunila, John Volk**, and **John Karolus**, State of Connecticut, Department of Agriculture, Bureau of Aquaculture, P.O. Box 97, Milford, CT 06460.

Vibrio parahaemolyticus is an enteric pathogen transmitted to humans primarily through consumption of raw or mishandled seafood. Like other members of the genus *Vibrio*, it is a gram negative, halophilic bacterium that occurs naturally in estuarine environments. In the late summer of 1998, an outbreak of *Vibrio parahaemolyticus* was epidemiologically linked to a shellfish bed on the northern shore of Long Island, NY. Based on an Interstate Sanitation Shellfish Conference (ISSC) 1998 Interim Control Plan for *Vibrio parahaemolyticus*, routine monitoring was required at this location and other locations where outbreaks of *Vibrio parahaemolyticus* had occurred. FDA scientists at the laboratory in Dauphin Island, Alabama developed rapid DNA probe procedures for the detection of *Vibrio parahaemolyticus* and for the detection of the human pathogenic form of this bacterium.

Briefly, the procedure requires oysters to be homogenized with alkaline peptone water (1:1). Aliquots are then placed on T_1N_3 agar plates for overnight growth at 35° C. Whatman 541 filters are applied on the surface of the agar plate for adhesion of the bacterial growth to the filter. The bacterial colonies are lysed, followed by DNA splitting. Then the alkaline phosphatase labeled DNA probe hybridization occurs. After washing, the filters are placed in an NBT/BCIP solution which will precipitate dark blue dyes in the presence of the DNA bound alkaline phosphatase. Blue black spots representing the *Vibrio parahaemolyticus* colonies are counted for a result.

The training of individuals from eight different states in these procedures resulted in the development of an FDA-ISSC partially funded national oyster surveillance program. Each state is testing oysters from two locations along their shore for twelve months. All testing is performed at the individual state laboratories. CT had started testing the first week of June 1999 and New York started in August 1999. *Vibrio parahaemolyticus*

has been found in CT and NY oysters. Results have shown greater levels of the bacterium in the summer when the water temperature is higher. When the water temperature dropped below 16–17 °C, *Vibrio parahaemolyticus* could not be detected. No forms of the bacterium pathogenic to humans have been found. Results from all states are being sent to the FDA Laboratory in Alabama for analysis.

EXPLORING DIVERSITY THROUGH AQUACULTURE. **Julia Rankin, Michael Wilcox** and **Donald Harris**, AgriScience & Technology Center, Bloomfield, CT 06002.

Exploring Diversity Through Aquaculture (EDA) involves 40 racially diverse students from 8 districts in the Greater Hartford area. This number reflects 20% deduction in proposed numbers due to a 25% reduction in funding. These students have successfully completed the first two weeks of this scientifically intensive four-week residency program which began in July 1999 and will be completed in late June 2000. The first week utilized the resources of the University of Connecticut, ending with an overnight trip to coastal Rhode Island. The second week, also in July, explored the fisheries and aquaculture industries of Maine, beginning in Eastport, the easternmost point in the continental United States, and finishing in Bath, in southern Maine. Students used the University of Maine at Machias and Husson College in Bangor as home bases. The first two weeks were tremendously rewarding, as students forged lasting friendships across racial and cultural boundaries while constantly exploring myriad aspects of science, often in a hands-on environment to which they would not normally be exposed. The third week of the program will occur later in 2000, with the same group of students exploring warm-water aquaculture at Auburn University in Alabama in April and studying the fisheries of the Gulf of Mexico. The final week of the program will allow students to study the burgeoning aquaculture history of Jamaica. At each location, students have been and will be given the opportunity to explore the cuisine and culture specific to each region, through interviews and festivals. Students are also preparing on-going portfolios featuring CAPT-based activities that focus on the scientific, sociological, economic and anthropological aspects of each activity in the program.

ECOLOGICAL SIGNIFICANCE OF THE PROVIDENCE RIVER QUAHOGS: POPULATION FILTRATION. **Michael A. Rice**, Department of Fisheries, Animal and Veterinary Science, University of Rhode Island, Kingston, RI 02881; **April Valliere, Mark Gibson** and **Arthur Ganz**, Rhode Island Division of Fish and Wildlife, Coastal Fisheries Laboratory, 1231 Succotash Rd., RR#1, Wakefield, RI, 02897.

Filter feeding by populations of bivalves has been suggested as a means of reducing eutrophication in coastal estuaries by exerting control of phytoplankton populations in the water column. Frequently, large populations of mature shellfish residing behind pollution closure lines in estuaries represent a large filter feeding biomass. The standing crop of quahogs, *Mercenaria mercenaria*, in the Providence River averages 9.1 clams/m^2 or about 26,400 tonnes, filtering about 1.05×10^7 m^3 of water daily or a rate equivalent to 21% of the rate of water exchange during a tide cycle. Due to annual temperature effects, population filtration ranges from 0 in the winter to 2×10^7 m^3/day in August. The population of quahogs, however, is composed of mostly older adults with valve lengths in excess of 60 mm. These large animals are slow growing, have a low rate of secondary production in relation to standing crop biomass, and have a neutral nitrogen balance (organic-N assimilated = NH_3-N excreted). These large adults increase sedimentation through filter feeding, but since they are neither harvested nor growing they do not directly remove much nitrogen from the system, although the increased sedimentation rates may result in increased sediment denitrification. Filtration by the standing crop of quahogs may remove 76.2 tonnes of organic nitrogen from the estuary annually by depositing it to the benthos. Harvest of quahogs at MSY can remove 8 tonnes of organic nitrogen annually. As part of a Narragansett Bay wide shellfisheries management plan, 10% of the standing crop of quahogs in the Providence River is recommended for relay to management beds down bay for later harvest. Smaller more rapidly growing quahogs have the capability of incorporating organic nitrogen into growing tissues and, if harvested regularly, provide a mechanism for direct removal of nitrogen from the estuary. The removal of quahogs from the dense assemblages in the Providence River reduces the population filtration by only 10%, but it culls the population making room for faster growing juveniles and small adults. This is publication 3785 of the Rhode Island Agricultural Experiment Station, University of Rhode Island.

COMPARATIVE EVALUATION OF THE MULTIPLEX PCR WITH CONVENTIONAL DETECTION METHODS FOR *HAPLOSPORIDIUM NELSONSI* (MSX) *HAPLOSPORIDIUM COSTALE* (SSO), AND *PERKINSUS MARINUS* (DERMO) IN THE EASTERN OYSTER, *CRASSOSTREA VIRGINICA*. **Spencer Russell, Soledad Penna** and **Richard French**, University of Connecticut, Dept. of Pathobiology, 61 North Eagleville Rd, U-89, Storrs, CT 06269.

Presently, the monitoring of cultured oyster populations for pathogens is infrequent due to the dependence on traditional, time consuming diagnostic assays. A multiplex polymerase chain reaction (MPCR) has been developed which rapidly detects the proto-

zoan parasites, *Perkinsus marinus* (Dermo), *Haplosporidium nelsoni* (MSX) and *Haplosporidium costale* (SSO), which infect the cultured oyster, *Crassostrea virginica*. Conventional diagnostic methods (histopathology and Ray/Mackin fluid thioglycollate assay) for *H. nelsoni*, *H. costale* and *P. marinus* respectively were compared and evaluated with the MPCR. Ninety-one adult oysters were collected from randomly selected beds in Westport, CT, (n = 37) and Milford, CT (n = 54) and subjected to all three assays. The Ray/Mackin assay detected *P. marinus* infections in 59 of 91 (64%) oysters and MPCR revealed infections in 73 of 91 (80%) oysters. Histological examination detected 37 of 91 (40%) oysters infected with *Haplosporidium* plasmodia. The MPCR was able to differentiate between the two *Haplosporidium* plasmodia, detecting 9 of 91(10%) oysters infected only with *H. nelsoni*, 37 of 91 (40%) oysters with only *H. costale*, and 32 of 91 (35%) oysters with mixed infections of *H. nelsoni* and *H. costale*. These results indicate the MPCR is a more sensitive assay for the detection of *P. marinus* and is able to detect and differentiate between the two *Haplosporidium* species. This would suggest that the MPCR can be useful at low infection intensity by being able to detect pathogens, based on pathogen DNA concentrations as low as 10fg., for *H. nelsoni* and 1pg. for both *H. costale* and *P. marinus*.

IDENTIFICATION OF A PROTOZOAN PARASITE IN THE AMERICAN LOBSTER, *HOMARUS AMERICANUS*, FROM LONG ISLAND SOUND. **Spencer Russell, Kristen Hobbie, Tom Burrage, Claudia Koerting, Sylvain De Guise, Salvatore Frasca Jr.,** and **Richard A. French**, University of Connecticut, Department of Pathobiology, 61 North Eagleville Rd. U-89, Storrs, CT 06269.

Mortalities of the American lobster, *Homarus americanus*, in Long Island Sound have severely increased and as a result are critically damaging the regional lobster industry. Necropsies were performed on 75 individual lobsters collected from six different locations in Long Island Sound. Gross observations found in 'sick' lobsters included a pink discoloration to the ventral surface of the abdomen (tail meat) and lethargic/limp behavior. An associated coagulopathy of hemocytes is also observed in affected lobsters. Initial bacteriology findings include isolation of *Vibrio* spp. and spirochetes. No *Aerococcus* have been isolated to date. Histologic examination has been conducted on various tissues, including heart, gill, hepatopancreas, antennary glands, intestine, muscle, exoskeleton, eyes, antennae, and central nervous system. The histopathology is consistent with a systemic inflammatory disease affecting multiple tissues but primarily the nervous system. Associated with lesions is a protozoan parasite morphologically characterized as an amoeba, tentatively *Paramoeba* sp.

LIVING CONTAMINANTS IN MICROALGAL FEED PRODUCTION TANKS – WHAT DO WE DO NOW? **Barry C. Smith** and **Mark Dixon**, USDOC, NOAA, National Marine Fisheries Service, Northeast Fisheries Science Center, Milford Laboratory, Milford, CT 06460.

The Greenhouse for Research on Algal Mass Production Systems (GRAMPS) at the Milford Laboratory can produce 20,000 liters of dense algal culture per day. This is done by using half of two 20,000-liter tanks each day and refilling one while the other is being drained. Backup starter cultures for these tanks can be housed in up to eight 500-liter cylindrical tubes. Algal production can be reduced severely if any of these cultures become contaminated with unwanted organisms.

Contaminants that invade algal cultures lower the yield of algae, increase the cost of production, and sometimes destroy the culture. Some contaminants are benign in that they establish a minor population that has no detectable effect on the algal culture or on what the culture is used for. Other contaminants, such as many ciliates, algae of unsuitable nutritional value that outcompete the desired algae, and algae that are harmful to the animals to be fed, spell disaster for an algal culture. Routine quality control measures, such as microscopic observation, should be used to detect contaminants as early as possible. When a contaminant is identified in an algal culture, the fate of the culture must be assessed. Will the contaminant dominate or destroy the culture? Is it harmful to the use of the algae? Can the contaminant be tolerated? If the decision is made that the contaminant cannot be tolerated, the source of the contaminant must be located. There are several possible contamination vectors. Any flow, act, or event involving the algal cultures, or even in the facility, could be responsible. Perhaps the most common source of contamination is the treatment of the water used to fill the culture. Contaminants can get on an operators' hands, hoses, and other accessories that may contact, even briefly, the culture. Even a random splash from a nearby workstation can inoculate an algal culture with a contaminant.

Once the source of contamination is located, a remedy can be found and assessed economically. The best way to solve the problem of contaminants in an algal culture is to prevent them from entering the system; this sounds easier than it is. One consideration is the cost of increasing the level of filtration or pasteurization. Is treatment with ultraviolet light more desirable? Hygiene/microbiological sanitation is one way to reduce the risk of contamination. Some remedies have been as simple as switching the order of daily tasks performed by an operator so that possible contaminants do not contact the person until after the algal cultures have been cared for. Contaminants may become resident in the culture vessels or plumbing; once established, the only way to remove them is with microbiological sanitizing measures and tactics. Every surface must be sanitized in such a way that the system has been thoroughly cleaned and no surface has been recontaminated.

Living contaminants in an algal culture are best managed by an

hierarchical decision tree that 1) assesses the impact of the contaminant, 2) locates the source of harmful contaminants, and 3) evaluates and tests possible control measures to identify procedures that are effective and economical.

RECENT RESULTS FROM FIELD AND LABORATORY STUDIES OF QPX. Roxanna Smolowitz, Ernest Marks, and **Chris Brothers**, Marine Biological Laboratory, Woods Hole, MA 02543; **Dale Leavitt** and **Bruce Lancaster**, Woods Hole Oceanographic Institution, Woods Hole, MA 02543.

Field studies of clam disease (QPX) have been ongoing in Provincetown and Duxbury, MA since October, 1997. Data collected to date show development of visible QPX nodules in 38% of mixed parentage (wild/notata) hard clams (*Mercenaria mercenaria*) planted in Duxbury, MA in Oct. 1997. However, wild/notata, 100% notata and 100% wild parentage clams planted and sampled at the same times listed above from Provincetown, MA have shown no nodules to date. Clams from Duxbury are significantly larger than Provincetown clams, so decreased food quantity/quality does not appear to be a significant factor in the development of the disease. While the percentage of animals grossly positive in Duxbury was high during the Oct. 1999 collection period, no mortality was noted. It is expected that mortality will occur during the spring of 2000.

In the laboratory, raceways were constructed and put into use in August of 1999. Raceways consisting of a total of 32 individual containers received hard clams of approximately 30 mm in shell height that had been treated in one of five exposure methods (no exposure, saline only injection, QPX injection, exposure to QPX in the water column, exposure to QPX infected two year old adults gathered from Provincetown flats). Cultured QPX was used in the injection and water column exposures. To date, no significant mortality has occurred in the raceways.

EFFECTS OF VARIOUS MICROALGAL DIETS ON THE GROWTH AND SURVIVAL OF LARVAE OF SEA SCALLOPS, *PLACOPECTEN MAGELLANICUS*. *Bethany A. Starr*, Beals Island Regional Shellfish Hatchery, P.O. Box 83, Beals, ME 04611.

A series of feeding experiments using single and mixed species of unicellular microalgae to determine the growth and survival of *Placopecten magellanicus* larvae were conducted at the University of Maine at Machias Aquaculture Room from late August – November 1999. The following algal species were used: *Isochrysis galbana* (Tahitian strain), *Chaetoceros neogracile, Thallasiosira weissfloggi, Rhodomonas salina*, and *Tetraselmis chui*.

Experiments were conducted at 14 ± 1 °C; algal cultures were maintained at temperatures between 17–20 °C. The experiment was a random block design with five treatments of food combinations (n = 4). Experimental units consisted of 40 L aerated kriesels. In early trials, larvae were fed at a rate of 20,000 cells/ml for single algal species, and 10,000 cells/ml/species in mixed algal combinations. The feeding regime was later adjusted to begin at 5000 cells/ml with trocophores and increased by 5000 cells/ml at days 6, 11, 16, and 21 until 20,000 cells/ml were attained after which time this feeding density was maintained. A feeding trial was concluded when larvae reached the pediveliger stage or died. Growth and survival rates were determined by subsampling each kriesel on drain-down days (occurring every 2—3 days). Animals were measured using an ocular micrometer to determine growth rates.

Preliminary results indicate that sea scallop larvae grew best on a combination of *Isochrysis galbana* and *Tetraselmis chui*. This may be due to the high fatty acid content of the *T. chui*. Other preliminary results indicate that survival rates were stable early in the larval stage, but decreased as the larvae reached the pediveliger stage (depending on food treatment). Future research will focus on identifying the specific fatty acid content of each algal species, and also the assimilation of these algae by the scallop larvae.

OBSERVATIONS ON GROWTH AND SURVIVAL OF JUVENILE BAY SCALLOPS (*ARGOPECTEN IRRADIANS*) FROM GENETIC LINES UNDER DIFFERENT DENSITY AND HOLDING CONDITIONS. Sheila Stiles, Tasha Robinson, and **Joseph Choromanski**, USDOC, NOAA, National Marine Fisheries Service, Northeast Fisheries Science Center, Milford Laboratory, Milford, CT 06460.

Hatchery-reared juvenile bay scallops (*Argopecten irradians*) from mass-spawned foundation crosses for genetic selection were tested under various nursery and holding conditions to maximize growth and survival for breeding. Lines consisted of scallops of two initial mean sizes, MS99-2 (12.5mm) and MS99-4 (4 mm). Scallops from each line were divided into four groups under different holding conditions: laboratory trays at 2 different densities, a raceway tank, and a suspension dock at approximately 10 feet under water. The latter two groups were in pearl nets. Scallops were measured for length and their volume estimated weekly over a five-week period during the summer.

Overall survival was high and growth was significant for most of the scallops. Best growth for the smaller-sized scallops (MS99-4) initially occurred up to 10 mm in the flowing water trays, after which growth was better in the raceway tanks, and then finally was best at the dock site, with a mean size of 18 mm after 5 weeks. Survival was best in the trays and lowest at the dock. A few small crabs were observed in the pearl net with the scallops which accounted for the higher mortality there. The larger scallops (MS99-2) grew better in the raceway tank and the tray with less density, at the beginning of the experiment. However, by the end of the study, the scallops from the MS99-2 line at the dock were more than 2-fold larger (26.7 mm). Mean sizes of scallops held in the trays at the two densities were not very different for either line. No

line-specific performance was apparent. Results generally confirmed those of other researchers for the efficient production of bay scallops under different holding conditions at various nursery and growth stages.

PRINCIPAL DISEASES OF CONNECTICUT'S OYSTERS. **Inke Sunila, Joseph DeCrescenzo, John Karolus,** and **John Volk**, State of Connecticut, Department of Agriculture, Bureau of Aquaculture, P.O. Box 97, Milford, CT 06460.

Oysters are long-lived, sessile animals, which have the ability to accumulate hundredfolds of micro-organisms and pollutants while filter feeding. This makes them susceptible to diseases. Several factors can induce pathological changes in oysters: infections caused by viruses, bacteria and parasites, age, fouling, predation, siltation, biotoxins, starvation, pollutants, oxygen deficiency and variations and extremes in temperature and salinity. These factors may cause pathological changes such as inflammatory responses (acute or chronic), degenerations (vacuolization, inclusions, ceroidosis), cell and tissue death (necrosis, apoptosis), growth derangements (hyperplasia, metaplasia), hemodynamic and fluid derangements (edema, hemorrhage), and neoplasia (benign or malignant). The sum of environmental stimuli, together with the genetic make up of the oysters, will determine their likelihood for disease.

From 1997 to 2000 we collected 3000 oysters from a hundred sampling stations in Connecticut, processed them for histology and diagnosed them for different categories of pathology. Despite the tradition of transplanting oysters, different areas of CT's oyster grounds were characterized by different conditions. Fouling organisms such as *Crepidula* spp. occurred in the western and central part of CT and were replaced by limpets in the eastern part. Ampharetid worms, *Sabellaria vulgaris*, calcareous tubeworms and different species of Bryozoa were present in the central and western part of CT. Parasitic infestation by trematodes and Turbellaria were prominent in the eastern part. *Nematopsis ostrearum* was present at high prevalence from New Haven to Westbrook. *Polydora websteri* occurred in seed beds in the Housatonic, Quinnipiac and Thames Rivers and also in small rivers between Guilford and Westbrook. *Cliona* spp. and ciliates were present in all sampling stations. MSX-disease (caused by *Haplosporidium nelsoni*) occurred at epizootic prevalence causing high mortalities in western and central CT. Infected animals had chronic hyaline hemocyte inflammatory responses. Dermo-disease (caused by *Perkinsus marinus*) established high prevalence on the entire coastline. Infected specimens were likely to have ceroidosis. Ulcers were found in the intestine and stomach epithelia in oysters from Bridgeport, Norwalk and the Housatonic River. Xenomas in the gills and viral gametocyte hypertrophy occurred at low prevalence in all sampling areas. Both benign and malignant tumors were detected. A large pericardial tumor, described as a vesiculo-epithelial polyp, was found in one oyster in Groton. Several cases of enteric adenocarcinoma *in situ* were detected. Tumors were composed of cystic glands with cellular debris and mucus inside the lumens. Epithelia forming the glands were basophilic, thickened and with increased cellularity and mitotic figures. According to published reports, similar lesions have been induced in the laboratory by exposing oysters to sediment from Black Rock Harbor, Bridgeport.

In conclusion, disease resistance to MSX is developing in the oyster population. The overall health of the Connecticut oyster can be considered good.

ESTABLISHMENT OF RHODE ISLAND'S FIRST COMMERCIAL SHELLFISH HATCHERY. **Karin A. Tammi, Wayne H. Turner,** and **Luning Sun**, Hope Shellfish Company, Post Office Box 4, Portsmouth, Rhode Island 028711; **Michael A. Rice**, Fisheries, Animal and Veterinary Science Department, University of Rhode Island, Kingston, Rhode Island 02811.

Hope Shellfish Company (HSC) recently completed permitting to become the first commercial shellfish hatchery in the history of Rhode Island. This project has been four years in the making and will be operational in the spring of 2000. The principals of the HSC have invested a considerable amount of time, patience and energy securing the financing and permits. Traversing the recent aquaculture regulations promulgated by the state's Department of Environmental Management and the Coastal Resource Management Council were not easy tasks. In order to acquire the proper approvals, Hope Shellfish Company networked with more than 25 regulatory representatives from federal, state and local agencies. Explaining the aquaculture project to regulatory agencies required preparation of detailed and lengthy applications with the total amount of documentation easily exceeding 500 pages.

By combining years of experience in shellfish restoration and commercial culturing, the principals anticipate producing over 40 million large-size shellfish seed annually when the facility is fully operational. HSC will begin producing bay scallops, *Argopecten irradians*, eastern oysters, *Crassostrea virginica*, quahogs, *Mercenaria mercenaria*, and soft-shell clams, *Mya arenaria*. It was estimated that this project could generate enough "raw product" to allow 500 fishermen to earn a competitive day's pay year-round and could support jobs in value-added industries such as processing, marketing, and distribution and will have a positive influence on recreational shellfishing and tourism in Rhode Island. This facility offers tremendous opportunities to aquaculturists, shellfishermen, researchers, educators, and resource managers in the State of Rhode Island and beyond. The benefits of this project are numerous, yet none more significant than the overall economic and cultural benefits to the citizens in this region. This project has the ability to elevate the status of aquaculture in the State as well as to educate students and researchers from the region's schools and universities.

AN INEXPENSIVE DIGITAL TEMPERATURE SENSOR FOR DATA ACQUISITION USE IN AQUACULTURE. **James C. Widman, Jr.,** USDOC, NOAA, National Marine Fisheries Service, Northeast Fisheries Science Center, Milford Laboratory, Milford, CT 06460.

A few parts, a personal computer, and some mechanical ability can yield an inexpensive digital thermometer which can be used for monitoring air and water temperatures in an aquaculture facility. Digital temperature sensors (DS1820, DS18B20) and COMM port adapters (DS9097U) are available from Dallas Semiconductor at http://www.dalsemi.com. The temperature sensors have an accuracy of ± 0.5 °C from −10 to 85 °C and a full range of −55 to 125 °C. By using category-5 cable, sensors can be placed at a distance of up to 300 meters from a personal computer. After soldering the cable to the sensor, a small piece of vinyl tubing is placed over the wire and attached to the body of the sensor with underwater epoxy or silicone. To ensure no electrical leakage, the entire surface of the sensor is coated. This adds slightly to the thermal mass and increases response time, but temperatures generally change slowly in an aquaculture facility. These sensors have been used for over a year in a submerged marine environment.

RESPONSES OF BAY SCALLOPS, AT SEVERAL LIFE-HISTORY STAGES, TO CULTURES OF POTENTIALLY-HARMFUL MARINE MICROALGAE. **Gary H. Wikfors, Jennifer H. Alix, Sara Barcia,** and **Julie Cullum,** USDOC, NOAA, National Marine Fisheries Service, Northeast Fisheries Science Center, Milford Laboratory, Milford, CT 06460; **Sandra E. Shumway,** Southampton College, LIU, Southampton, NY 11968; **Roxanna M. Smolowitz,** Marine Biological Laboratory, Woods Hole, MA 02543.

Widespread use of the term "Harmful Algal Bloom" begs the question: Harmful to whom? Molluscan shellfish have been recognized as vectors of microalgal toxins to human consumers for millennia, but detrimental effects of some microalgae upon the mollusks themselves have received less attention. As part of a larger study designed to investigate the role of grazing in the bloom dynamics of microalgae for which there is some evidence of grazing suppression, we conducted experimental exposures of bay scallops, *Argopecten irradians*, at several life-history stages (embryos, larvae, post-set, and juveniles) to a number of cultured microalgal strains. Microalgae investigated included: 1) dinoflagellates, two strains of *Prorocentrum minimum*, *Gyrodinium aureolum*, and *Gymnodinium splendens*; 2) a raphidophyte, *Heterosigma carterae*; and Prymnesiophytes, two strains of *Prymnesium parvum* and one of *P. patelliferum*. Scallop response variables measured included survival, growth, development, feeding behavior, and histopathology. Effects ranging from subtle and sublethal to acute toxicity were observed. The most dramatic, lethal effects were seen with a new strain of *Prorocentrum minimum*, collected by Dr. Patricia Glibert from a 1998 bloom in the Choptank River, MD, and with a new strain of *Prymnesium parvum*, isolated by Dr. Robert Guillard from Boothbay Harbor, ME. In addition to limiting the harvest of molluscan shellfish for human consumption, harmful algal blooms have the potential to affect the population biology of molluscs themselves.

PROCESS DESIGN FOR ARTEMIA CULTURE AT COASTAL BIOMARINE. **Loy Wilkinson.** Coastal BioMarine, 250 Northrup St., Bridgewater, CT 06752.

A method for the design of a closed cycle system for the intensive production of *Artemia* from algae is described. The process for the intensive production of *Artemia* is an adaptation of the one practiced at the Laboratory of Aquaculture and Artemia Reference Center, University of Ghent and described in the Manual of the Production of and Use of Live Food for Aquaculture. The overall material balances are presented as well as the design method for individual equipment in the process. Emphasis is placed on the treatment of the water to remove dissolved organics and ammonia. Theoretical and in-practice parameters for effective removal of these contaminants by means of a trickle bed filter are discussed.

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ABSTRACTS OF TECHNICAL PAPERS

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CONTENTS

GENETICS AND BREEDING

INVERTEBRATE FISHERIES

SHELLFISH BIOLOGY

SHELLFISH HEALTH MANAGEMENT

ENVIRONMENTAL AND ANTHROPOGENIC INFLUENCES ON SHELLFISH

PCB ASSIMILATION IN OYSTERS (*CRASSOSTREA VIRGINICA*): AN IMPLICATION FOR REPRODUCTIVE IMPAIRMENT. **Fu-Lin E. Chu,* Philippe Soudant,** and **Robert C. Hale,** Virginia Institute of Marine Science, School of Marine Science, College of William and Mary, Gloucester Point, VA 23062.

Polychlorinated biphenyls (PCBs) are of concern, in part due to their high persistence and bioaccumulation potential. This may be particularly acute for filter feeders due to association of PCBs with natural particulate organic matter. Two experiments were conducted to examine: (1) PCB assimilation in reproductively active oysters fed daily with 0.1 g PCB-sorbed algal paste containing 0, 0.1 or 1.0 g PCBs (mixture of Aroclor 1242, 1254, and 1260) for 15 and 30 days; and (2) PCB assimilation and its impact on reproduction in conditioned oysters fed, prior to gametogenesis, daily 0.7 g PCB-sorbed algal paste containing 0, 0.35 or 3.5 g PCBs. Changes in lipid and fatty composition in oyster tissues and gametes were also analysed. Results revealed that: (1) PCB accumulation was dose and time dependent and tissue (organ) specific; (2) Higher PCB contents were found in organs (gonad and visceral mass) rich in reserve lipids than adductor muscle and gills, which are dominated by structural lipids; (3) PCBs were transported to eggs, which had PCBs contents ranged from 247 to 671 ng PCBs/g tissue DW; (4) PCB exposure reduced slightly the weight percentage of certain polyunsaturated fatty acids (20:4n-3, 20:5n-3 and 22:6n-3) in eggs; (5) After exposure to 3.5 g PCBs daily for 8 weeks, structural lipids (e.g., phospholipids) in gonad, adductor muscle and mantle decreased, while triacylglycerol (TAG) increased in digestive gland; and (6) Compared to controls, PCB exposure resulted in fewer females and spawned females in conditioned oysters. Impairments of structural lipid synthesis and the mobilization/transport of reserve lipids, particularly TAG, are believed to be the cause of the delaying and/or inhibition of oogenesis in PCB-exposed oysters.

ADHERENCE AND INVASION MECHANISMS OF VIBRIO VULNIFICUS WITH OYSTER AND FISH CULTURED CELLS. **Gaskov Clergé, Mahendra H. Kothary, Marianne D. Miliotis, Darcy E. Hanes, Seynabou Fall, Jeffrey W. Bier, Dhirendra B. Shah,** and **B. D. Tall,*** JIFSAN, US FDA, Washington, D.C. 20204; **Broderick Eribo,** Howard Univ., Washington, D.C. 20059; **Jerome F. La Peyre** and **Mohamed Faisal,** VIMS, Gloucester Point, VA 23062.

Vibrio vulnificus (*Vv*) causes systemic infections in many seafood hosts; in humans it causes gastroenteritis, wound infections, and septicemia. To investigate the pathogenic mechanisms involved, we examined several strains by electron microscopy (EM) for presence of adherence factors. These studies revealed 3.5 nm fibrillar structures composed of linear strands, multiple strand bundles or wiry aggregates radiating from the bacterial surface. Using a KSCN/$(NH_4)_2SO_4$ precipitation procedure, we obtained a crude fibrillar extract (CFE) which consisted of single filaments, filaments in bundles, which also possessed hemagglutination (HA) activity. CFE obtained from both biotypes of *Vv* hemagglutinated sheep, chicken, bovine, human O and eel RBCs. However, CFE obtained from biotype 1 cells only hemagglutinated human A and B RBCs. Maximal expression of the adhesin occurred when cells were grown for 18 h on Thiaproline-NaCl-Glutamate-Agar adjusted to a pH 6 or 8, and incubated at 30 °C. Cells grown at a pH of 7.4 or at a temperature of 17° or 37 °C, or under anaerobic conditions were HA negative. These results suggest that expression of this fibrillar adhesin is controlled by environmental signals. To determine its role in adherence and invasion, Tn*phoA* mutagenesis was carried out and transconjugants were screened for lack of HA activity. Comparison of these mutants with the parental strain in adherence and invasion assays with primary oyster mantle, heart, intestinal, and hemocyte cells demonstrated that adhesin expression is needed for adherence and invasion into primary oyster cells. Studies performed with Mummichog (*Fundulus heteroclitus*) primary anterior kidney and liver cells showed similar results. However, different cell affinities were noted. To further investigate the invasion mechanisms involved, uptake assays were performed with Atlantic menhaden liver (AML) cells. Results from these studies suggest that uptake of *Vv* occurs at a limited number of sites. However, invasion efficiency of the afibrillated mutants was not significantly different from that of the parental strain suggesting the existence of host receptor differences among primary and immortalized cultured cells or that more than one ligand may be involved in the invasion of *Vv* into AML cells. Inhibitors of actin, microtubulin, and receptor-mediated endocytosis showed that invasion of stationary grown *Vv* was dependent only on the microtubulin pathway. However, uptake of log phase (LP) grown *Vv* was dependent on both actin and microtubulin suggesting that bacterial ligand expression differs with infective growth stage of *Vv*. Studies focused on the role of signal transduction in invasion of LP grown cells showed that the activation of s-protein tyrosine kinase (sPTK) and protein kinase C (PKC) are involved in *Vv* entry into AML cells. If invasion and cytotoxicity are sequential events, then inhibitors that block invasion should also reduce cytotoxicity. Using lactate dehydrogenase (LDH) release, a stable cytosolic enzyme as a measure of cytotoxicity in inhibitor experiments showed that inhibition of uptake by colchicine (microtubulin inhibitor) did not prevent the release of LDH and, hence, did not reduce cytotoxicity. In contrast, inhibition of protein kinase activity caused a significant decrease in release of LDH suggesting that protein kinase activity is involved in *Vv*-mediated cytolysis, and invasion and cytotoxicity are two mutually exclusive events. In summary, these data provide evidence indicating that uptake of *Vv* differs among cultured cells; occurs at a

limited number of sites on the AML cell surface; was dependent on expression of bacterial surface ligands and on the involvement of host cytoskeletal elements and protein kinase activities. These data also indicate that *Vv* uptake and bacterial-mediated cytotoxicity of AML cells are independent events.

STRESS PROTEIN (HSP70) RESPONSE IN OYSTERS *CRASSOSTREA VIRGINICA* EXPOSED TO VARIOUS STRESS AGENTS. **Luis A. Cruz-Rodríguez,* Fu-Lin E. Chu,** and **Philippe Soudant,** Virginia Institute of Marine Sciences, School of Marine Sciences, College of William and Mary, Gloucester Point, VA 23062.

Application of stress proteins (or heat shock proteins) as cellular biomarkers of exposure to environmental pollutants have been investigated in many aquatic organisms. This study investigated the stress protein (Hsp70) response to various stress agents (contaminated sediments, PCBs, and Cd^{2+}) in oysters (*Crassostrea virginica*). Oysters were exposed to 0, 1, 1.5, or 2 g contaminated sediments (CS) for 5, 10, 20, and 40 days. A significant increase in Hsp70 response was noted after 40 days exposure compared to non-exposed oysters. Oysters exposed to 2 g CS showed the largest increase in Hsp70, but no dose dependency in the response was noted. Reproductively active oysters fed 0.1 g PCBs-sorbed algal paste daily containing 0, 0.1, or 1.0 μg PCBs for 15 and 30 days showed a decrease in Hsp70 in oysters exposed to 1 μg PCBs for 15 days. This reflected total soluble protein trends. No significant difference in Hsp70 levels was observed in those exposed for 30 days, although an increasing trend was noted. Reproductive inactive oysters fed 0.7 g PCBs-sorbed to algal paste daily containing 0, 0.35, or 3.5 μg PCBs for 8 weeks with or without 0.3 g artificial sediments added, showed no significant increases in Hsp70. However, those exposed to the additional 0.3 g artificial sediments showed increases in the Hsp70 levels compared to those without. The absolute value in the magnitude of the response observed is greater in those exposed to sediments and PCBs than to sediments alone. Oysters exposed to 0, 15, or 25 ppb Cd^{2+} showed a significant increase in Hsp70, but no dose dependency was noted. Generally, in oysters as in other organisms, the stress protein response is elicited in instances where the stress agent causes protein damage. Thus, stress proteins can be used in oysters as an indicator of exposure to proteotoxic stress.

TEMPERATURE AND RANGE EXTENSION BY *PERKINSUS MARINUS*. **Susan E. Ford,[1]* Roxanna Smolowitz,[2]** and **Marnita M. Chintala,[1,3]** [1]Haskin Shellfish Research Laboratory, Rutgers University, Port Norris, NJ 08349; [2]Marine Biological Laboratory, Woods Hole, MA 02543; [3]Atlantic Ecology Division, U.S. EPA, Narragansett, RI 02882.

Between 1990 and 1992, Dermo disease of oysters, caused by *Perkinsus marinus*, experienced a 500-km northward range extension and is now established as far north as Massachusetts. Climate warming during the 1980s and early 1990s, combined with historical introductions of infected oysters, has been hypothesized as the cause. Surprisingly, anecdotal reports of oyster growers indicated that the disease was causing few deleterious effects in the Northeast. To document and investigate possible causes for this assertion, we monitored numerous oyster stocks between Delaware and Cape Cod Bays to describe disease cycles, to measure Dermo effects on oysters, and to compare results with data from more southern regions. We also investigated whether a low-temperature-tolerant strain of *P. marinus* is now present in the Northeast. Results of this two-year study showed that *P. marinus* behaves in its new range very much as it does in southern areas where it has been enzootic for decades. Seasonal cycles are similar, as is the 2–3 year progression to a full epizootic. Mortality during the present study was a least as great as in the south. Temperatures in most of the growing areas examined readily became warm enough to sustain high *P. marinus* proliferation and winters were not cold enough to limit disease cycles. Data from an *in vitro* growth assay of *P. marinus* isolates from North Carolina to Massachusetts, suggest responses to temperature that vary along a latitudinal cline; however, there was no consistent evidence from this assay, or from *in vivo* proliferation, that a low-temperature-tolerant strain has invaded the Northeast.

RESPONSES OF OYSTERS AND THEIR HEMOCYTES TO CLINICAL AND ENVIRONMENTAL ISOLATES OF *VIBRIO PARAHAEMOLYTICUS*. **Fred J. Genthner*** and **William S. Fisher,** US EPA, Gulf Breeze, FL 32561; **Aswani K. Volety,** Florida Gulf Coast University, Fort Meyers, FL 33965; **Ben D. Tall** and **Sherill K. Curtis,** JIFSAN, US FDA, Washington, D.C. 20204; **Susan A. McCarthy,** US FDA, Dauphin Island, AL 36528.

Interactions of *Vibrio parahaemolyticus* with oysters and oyster hemocytes were studied using three environmental isolates (1094, 1163 and ATCC 17802) and three clinical isolates (2030, 2062, 2107). Clinical isolates were from patients who became ill during the June 1998 food poisoning outbreak involving oysters from Galveston Bay in Texas. Environmental isolates were from oysters, crabs or sardines. All *V. parahaemolyticus* isolates possessed the thermolabile direct hemolysin (*tlh*) gene; only the clinical isolates had the thermostable direct hemolysin (*tdh*) gene (a putative virulence determinant). The capacity of oyster hemocytes to kill each *V. parahaemolyticus* isolate was examined *in vitro* using a novel dye reduction assay. Differences in killing by oyster hemocytes existed between and among environmental and clinical isolates. On average, environmental isolates were more susceptible to hemocyte killing than clinical isolates. Clinical isolate 2062 was more susceptible to killing by oyster hemocytes than the other two clinical isolates (2030, 2107) and displayed the most diffuse colony morphology on nutrient agar plates. Also, unlike the other two isolates, it lacked identifiable Alcian Blue stabilized capsular material that appears as irregularly distributed, spike-like, electron-dense deposits often observed spanning gaps between cells.

Additional experiments showed that when oysters were exposed to mixtures of a clinical (2030) and an environmental (1163) isolate, higher numbers of the clinical isolate were found in tissue and hemolymph. The significance of this research is that differences in *V. parahaemolyticus* isolates are described that influence ways in which these bacterial pathogens interact with oysters.

SUMMER STRESS PROTEIN RESPONSES OF CULTURED PACIFIC OYSTERS: DOES CHRONIC STRESS REDUCE TOLERANCE? Amro M. Hamdoun,[1] Daniel Cheney,[2] Ralph Elston,[2] Brian McDonald,[2] and **Gary N. Cherr,[1]** [1]Bodega Marine Laboratory, University of California Davis, Bodega Bay, CA 94923 and [2]Pacific Shellfish Institute, Olympia, WA 98501.

Pacific oysters (*Crassostrea gigas*) cultured in South Puget Sound routinely experience mass mortalities during the summer months. One factor thought to be associated with 'summer mortality' events is the combination of acute and chronic thermal stress often experienced during the summer. The responses of *C. gigas* to acute thermal stress have been well characterized. Briefly, these include induction of several members of the heat shock protein (HSP) 70 family and associated 'thermotolerance' to otherwise lethal temperatures. In contrast, relatively little is known about stress protein responses of chronically stressed Pacific oysters in culture. We tested the hypothesis that this summer stress alters the ability of Pacific oysters to mount normal heat shock responses. Neither constitutive nor inducible members of the HSP 70 family appear to be expressed at significantly elevated levels during the summer months. Moreover, oysters from one culture site did not induce HSP 69 after sublethal heat shock, for the duration of the summer. Most oysters were able to acquire thermotolerance after sublethal heat shock. However, some appeared to have already acquired some degree of thermotolerance prior to sublethal heat shock. Additionally we found that the normal stress protein response can be similarly inhibited by exposure to moderate levels of xenobiotics such as chromium. Thus, we suggest that oysters encountering natural and/or anthropogenic stressors in the field may acquire tolerance to chronic stress, but may lose the ability to mount a functional, rapid stress response to elevated temperature.

IMMUNE RESPONSES OF TWO SPECIES OF MUSSELS (*MYTILUS CALIFORNIANUS* AND *MYTILUS GALLOPROVINCIALIS/TROSSULUS* HYBRID) TO POLLUTANTS IN SAN FRANCISCO BAY, CA. Allison C. Luengen,* ETOX Department, University of California at Santa Cruz, Santa Cruz, CA 95064; **Carolyn S. Friedman,** Bodega Marine Lab, Bodega Bay, CA 94923; **A. R. Flegal,** ETOX Department, University of California at Santa Cruz, Santa Cruz, CA 95064.

Since mussels (*Mytilus californianus*) are routinely deployed in San Francisco Bay, California to monitor concentrations of contaminants in their tissues, this study was initiated to determine whether the elevated concentrations of some of those contaminants correlated with measures of variations in their immune response. Preliminary data from the latter measurements indicate that mussels from relatively contaminated sites exhibit elevated immune responses when compared with mussels from relatively pristine sites. This includes the following immune parameters: (1) number of hemocytes, (2) percentage of cells that phagocytosed particles, and (3) a phagocytic index, which describes how many particles were engulfed by phagocytic cells. Additionally, *M. californianus*, which does not live naturally in the Bay, appeared to show elevated immune responses when compared to *M. galloprovincialis/trossulus* hybrids that are endemic to the Bay. This disparity also indicates that *M. californianus*, which has been the species historically deployed as a biomonitor in the Bay, may not be the most appropriate species. Finally, this preliminary research has lead to the development of a new technique to evaluate phagocytosis in the mussel cell's hemolymph because the cells were too sensitive to the centrifugation step and the washing steps used in established methods.

RELATIONSHIPS BETWEEN OYSTER (*CRASSOSTREA VIRGINICA*) DEFENSE MEASUREMENTS AND TISSUE CONTAMINANTS. L. M. Oliver,*[1] W. S. Fisher,[1] A. K. Volety,[2] and **Z. Malaeb,[3]** [1]U.S. Environmental Protection Agency, National Health and Environmental Effects Research Laboratory, Gulf Ecology Division, 1 Sabine Island Drive, Gulf Breeze, FL 32561-5299. [2]College of Arts and Sciences, Florida Gulf Coast University, 10501 FGCU Blvd., Fort Myers, FL 33965-6565. [3]United States Geological Survey, Biological Resources Division, National Wetlands Research Center, Gulf Breeze Project Office, 1 Sabine Island Drive, Gulf Breeze, FL 32561-5239.

Bivalve mollusks such as *Crassostrea virginica* typically inhabit estuaries and coastal areas that are increasingly contaminated with anthropogenic chemicals. Oysters may bioaccumulate large quantities of metals, polyaromatic hydrocarbons (PAHs), and polychlorinated biphenyls (PCBs) without evident ill effects, but various measurements of bivalve defense activity show alterations from experimental chemical exposures and longer-term, field exposure to chemical mixtures. Forty oysters were collected from Bayou Chico and East Bay, two sites in Pensacola Bay, FL, known to differ in the type and magnitude of chemical contaminants. Tissue concentrations of metals, tri- and di-butylin (TBT, DBT), PAHs and PCBs were measured along with hemocyte number, phagocytic (PI) and bactericidal (KI) indices, and serum lysozyme and total protein levels. Hemocyte PI was significantly higher in East Bay oysters, which also had low tissue levels of PAHs, PCBs, TBT, DBT, and the metals Al, Cr, Fe, Ag, Cd, and Hg. Average hemocyte number, KI, serum lysozyme and protein were significantly higher in Bayou Chico oysters which also had high tissue concentrations of organic contaminants, butyltins, and Mn, Cu, Zn, and Sn. Canonical correlation analysis was used to examine rela-

tionships between tissue metals and defense measurements using linearly combined sets of variables. The highest possible correlation was positive: r = .934, between canonical variables composed of hemocyte number, PI, serum protein and lysozyme for defense, and Cd, Fe, Al, Pb, Zn, Mn, Sb, Ni, and Cr for metals. This suggestion of heightened defense activities in oysters from metal-contaminated sites is consistent with previous observations. The likelihood of complex relationships between oyster immune measurements and contaminant stress suggests that single chemical exposures and univariate analyses may be inadequate or misleading.

IN VITRO KILLING OF *PERKINSUS MARINUS* BY HEMOCYTES OF OYSTERS *CRASSOSTREA VIRGINICA*. **Aswani K. Volety,*** College of Arts and Sciences, Florida Gulf Coast University, 10501 FGCU Blvd, Fort Myers, FL 33907; **William S. Fisher,** US Environmental Protection Agency, Gulf Ecology Division, 1 Sabine Island Drive, Gulf Breeze, FL 32561.

A colorimetric microbicidal assay was adapted, optimized and used in experiments to characterize the capacity of eastern oyster (*Crassostrea virginica*) hemocytes to kill cultured isolates of *Perkinsus marinus*, a protozoan parasite causing a highly destructive disease of oysters throughout U.S. Atlantic and Gulf of Mexico coastal waters. *In vitro* challenges showed that hemocytes from two geographically distinct oyster stocks (Florida and Rhode Island) were able to decrease viable *P. marinus* cells by 45–52%. Variability in killing was most likely due to differences in susceptibility among the seven cultured isolates, which ranged in origin from Long Island Sound (CT) to Laguna Madre (TX). Hemocytes from oysters collected in Escambia Bay, FL, exhibited a relatively consistent mean killing capacity throughout a year-long period, averaging 57% across all months monitored with a range of 21–90%. Application of this technique demonstrated the *in vitro* capacity of hemocytes to kill *P. marinus*, but does not necessarily reflect their ability under natural conditions where the disease is widespread.

STRESS PROTEINS AS BIOMARKERS IN ESTUARINE SHELLFISH SPECIES. **Inge Werner,** School of Veterinary Medicine, Dept. of Anatomy, Physiology and Cell Biology, University of California at Davis, Davis, CA.

The application of stress proteins (or heat shock proteins) as cellular biomarkers of exposure to and/or effect of environmental pollutants has been proposed and investigated for a number of years. Members of this group of proteins are induced by a variety of stressors which either damage cellular proteins directly or cause cells to synthesize aberrant proteins. They have been detected in all organisms investigated, from bacteria and plants to humans, and are highly conserved across phyla. Functions include the stabilization of unfolded protein precursors before assembly, translocation of proteins into organelles, rearrangement of protein oligomers, dissolution of protein aggregates, and refolding or degradation of denatured proteins.

Numerous studies showed induction of hsp70 or hsp60 by laboratory exposure to chemicals which are known toxicants, e.g. heavy metals and several pesticides. Few studies, however, have examined the linkage of hsp induction to contaminant induced deleterious effect in the organism, or the hsp response to multiple stressors, chemical and physical, encountered in field situations. As more research is being conducted, new questions arise. Our laboratory has been investigating the suitability of hsp70 and hsp60 protein(s) in a variety of aquatic organisms as a field biomarker. Results indicate that the hsp response to stressors appears to be well suited as a biomarker of exposure and effect in some cases and not in others. This presentation will give insight into the pros and cons of using stress proteins as a biomarker in field studies, and tackle the question of whether induction of hsps indicates exposure to—and protection from potentially toxic compounds, or if it can predict deleterious effect in the organism.

FEEDING AND NUTRITION

EVOLUTIONARY AND FUNCTIONAL TRAJECTORIES OF THE BIVALVE GILL ABFRONTAL SURFACE: LESSONS FROM CONTEMPORARY CILIA AND MUCOCYTE DISTRIBUTIONS. **Peter G. Beninger,*** Laboratoire de Biologie Marine, Faculté des Sciences, Université de Nantes, 44322 Nantes Cédex France; **Suzanne C. Dufour,** Scripps Institution of Oceanography, University of California, San Diego, La Jolla, CA 92093-0202.

Recent data on the distributions of cilia and mucocytes on the bivalve gill abfrontal surface are analyzed with respect to evolutionary relationships of the principal Autobranch gill types. From the primitive function as a mucociliary cleaning surface in the Protobranchs, two evolutionary trajectories are evident: (1) progressive reduction of both cilia and mucocytes with resultant loss of surface function, seen in the homorhabdic filibranchs studied, and (2) reduction of cilia but retention or increase in acid mucopolysaccharide-secreting mucocyte density in the eulamellibranchs, corresponding to the assumption of a new function, probably in the reduction of frictional resistance to flow in the water canals. The heterorhabdic gill abfrontal surfaces present a mixture of these characteristics, corresponding to the staggered ontological and phylogenetic development of the two filament types: reduction of cilia and mucocytes on the ordinary filaments, retention of both on the principal filaments. The difference between heterorhabdic filibranchs and pseudolamellibranchs in degree of inter-lamellar fusion may be reflected in the functions of the retained mucocytes on the abfrontal surface of their respective principal filaments: reduction of resistance to water flow in the pseudolamellibranchs, lubrication for retraction of the gill during valve clapping for the

heterorhabdic filibranchs. Although the original function of the abfrontal surface has been rendered redundant by the various degrees of folding and fusion of the bivalve gill, the polyvalent potential of the mucocytes has resulted in the emergence of new functions.

A BIOCHEMICALLY-BASED MODEL OF THE GROWTH AND DEVELOPMENT OF PACIFIC OYSTER *CRASSOSTREA GIGAS* LARVAE. **Eleanor Bochenek,*** N.J. Sea Grant College Program, Sandy Hook Field Station, Fort Hancock, NJ 07732; **Eric Powell,** Haskin Shellfish Lab., Rutgers Univ., Port Norris, NJ 08349; **John Klinck** and **Eileen Hofmann,** CCPO, Old Dominion Univ., Norfolk, VA 23529.

A biochemically-based model was used to simulate the growth and metamorphosis of *Crassostrea gigas* larvae. This model, which is the first of its type, includes parameterizations of the metabolic costs associated with larval filtration, ingestion, and respiration. The initial biochemical content of the larva is determined by the composition of the egg. Changes in the initial ratios of protein, carbohydrate, neutral lipid and polar lipid occur as the larva grows in response to environmental conditions. The model obtains realistic larval life spans and success rates at metamorphosis under a range of environmental conditions based on a metabolic trigger of metamorphosis defined as a reduction in the ratio of storage products to structural components modulated by a reduction in filtration rate. Simulations show that larger eggs produce larvae that are more able to withstand poor food environments over the larva's life, suggesting that egg size is one variable accounting for the range of larval sizes at which metamorphosis is attempted and the success rate for metamorphosis. However, eggs can be too large. Optimal size, around 50 μm, yields greatest metamorphosis success. Other simulations show that food supply and environmental conditions also control the size range and success rate for metamorphosis by influencing the ratio of storage products (implemented as neutral lipid) to structural products (chiefly protein and polar lipid). For example, temperatures above 20 °C (and ≤30 °C) and salinities ≥20‰ (and ≤30‰) result in a large fraction of larvae successfully surviving metamorphosis. High temperature can spare low food supply up to a point because filtration rate increases with increasing temperature; however high food supply cannot spare low temperature.

USING *CHLORELLA* TO STUDY POSTINGESTIVE SELECTION IN BIVALVES. **Martha G. S. Brillant*** and **Bruce A. MacDonald,** Biology Department and Centre for Coastal Studies and Aquaculture, University of New Brunswick, Saint John, NB, Canada E2L 4L5.

Postingestive selection is known to occur in several species of bivalves, however the factors responsible for selection have not been established. We have shown that size and density of particles play a role in postingestive selection in the sea scallop (*Placopecten magellanicus*). Determining the role of particle chemistry or quality is more challenging. Particles chosen to study postingestive selection by chemical properties should appear physically identical but chemically distinct to the bivalve and must be traceable and quantifiable after passage through the bivalve. Also, the integrity of these particles within the bivalve stomach should be similar. We have addressed these factors by using *Chlorella* (CCMP 1227) to study postingestive selection. *Chlorella* has a thick cell wall and therefore can be killed by heat and remain intact. After heat treatment *Chlorella* has significantly lower carbon, nitrogen and photosynthetic pigments than fresh *Chlorella.* Scallops will be fed heat-killed algae and live algae simultaneously with one treatment labeled with ^{14}C. The feces will be collected and analyzed on a scintillation counter. Gut retention times of the two treatments will be compared to determine if postingestive selection has occurred. This method should provide a means of determining whether scallops can distinguish particles within the stomach on the basis of chemical properties alone.

NATURAL DIET EFFECTS ON FOOD UTILIZATION BY SEA SCALLOPS AND BLUE MUSSELS. **Peter J. Cranford,* Shelley L. Armsworthy, Michael J. White,** and **Timothy G. Milligan,** Fisheries and Oceans Canada, Bedford Institute of Oceanography, P.O. Box 1006, Dartmouth, NS, B2Y 4A2.

The widespread expansion of bivalve culture operations in estuarine and coastal systems is increasing the potential for bivalve filter feeders to affect regional trophic structure. It is therefore essential that bivalve food utilization be more fully comprehended to determine effects on coastal ecosystems and the sustainability of existing and expanding culture operations. Until recently, much of the information on bivalve functional responses to diet variability has been obtained using artificial diets (e.g. cultured algae) so that feeding conditions could be strictly controlled. However, recent studies on the responsiveness of feeding behaviour to natural diets have demonstrated the importance of conducting this work under more environmentally realistic conditions. In our paper, we will review recent studies we conducted on the effects of natural diets on food acquisition by *Placopecten magellanicus* and *Mytilus edulis* and the consequences to growth. The focus was on seeking generality on bioenergetic responses at sites characterized by low seston loads (<5 mg L^{-1}). Measured responses to ambient food supplies were used to construct hypothesis on: the effect of the different time-scales of variation in food supplies (hourly to interannually) on food acquisition processes; the relative importance of exogenous and endogenous forcing; the effect of particle flocculation on bivalve trophic resources and feeding behaviour; and the food utilization strategies of different bivalves.

ENHANCED PRODUCTION OF PACIFIC DULSE (*PALMARIA MOLLIS*) FOR CO-CULTURE WITH RED ABALONE (*HALIOTIS RUFESCENS*) IN A LAND-BASED SYSTEM. C. L. Demetropoulos* and **C. J. Langdon,** Hatfield Marine Science Center, Oregon State University, Newport, OR 97365.

Pacific dulse (*Palmaria mollis*) has shown itself to be a valuable algal feed for red abalone (*Haliotis rufescens*). Land-based tumble culture techniques capable of producing commercial quantities of *P. mollis* are still in development. An understanding of specific nutrient requirements and the importance of the relative velocities of algal rosettes to their culture medium are essential to increasing yields of *P. mollis*.

Under high photon flux densities, additions of a combination of nitrate and ammonia, as sources of nitrogen, to dulse cultures resulted in no significant difference in growth compared with additions of nitrate alone. A nitrogen to phosphorus (N:P) ratio of 11 provided the most economical use of phosphorus. Addition of trace metals, Fe, Mn, and Zn significantly increased dulse growth. Cultures supplied with a combination of both CO_2 and $NaHCO_3$ produced higher yields compared with those supplied with either inorganic carbon sources alone. Yields of *Palmaria mollis* were positively related to the relative velocity of rosettes to the culture medium, up to a relative velocity of 15 cm/sec.

NUTRITIONAL STATUS OF FOUR ALGAL DIETS FOR THE CAPTIVE CARE OF FRESHWATER MUSSELS. Catherine M. Gatenby* and **Daniel A. Kreeger,** Patrick Center for Environmental Research, Academy of Natural Sciences, Philadelphia, PA 19103; **Vanessa A. Jones,** and **David M. Orcutt,** Department of Plant Pathology and Weed Science, Virginia Tech, Blacksburg, VA 24061; **Bruce C. Parker,** Department of Biology, Virginia Tech, Blacksburg, VA 24061; **Richard J. Neves,** Virginia Cooperative Fish and Wildlife Research Unit, Department of Fisheries and Wildlife Sciences, Virginia Tech, Blacksburg, VA 24061.

The success of conservation efforts to restore dwindling freshwater mussel populations through culture and propagation requires an understanding of their nutritional requirements. We quantified key nutritional components of several freshwater algae to identify suitable diets for the care of unionid mussels in captivity. Total carbohydrate, protein, and lipid contents (% dry w/w) were compared among three green algae, *Neochloris oleoabundans, Bracteacoccus grandis, Scenedesmus* spp. and the diatom *Phaeodactylum tricornutum*, at different phases of growth: log, late log, stationary, and late stationary phase. We found no difference in protein content among species or growth phases; however, carbohydrate content was significantly greater in stationary phase green algae with *Scenedesmus* containing the most carbohydrate (50%). The greatest amount of lipid was found in log phase growth for all algae. Since the character of lipids is known to be of nutritional importance for bivalve molluscs, we also quantified and identified the fatty acid and sterol composition of these algae. On average, *N. oleoabundans* contained more fatty acids per mg of lipid (900 μg/mg lipid), and *B. grandis* contained more sterol per mg lipid (47 μg/mg lipid). The composition of fatty acids was similar among algae, with the exception that *P. tricornutum* contained greater amounts of C16:8, C20:0, C20:1, C20:3 and C22:5, and smaller amounts of C16:6. A greater percentage of unsaturated fatty acids was found at log phase than at stationary phase in all algae. The relative food value of these algae for supporting cultures of freshwater mussels will be discussed by comparing these data to measured rates of algae uptake and assimilation by the animals.

CHANGES IN THE FLUIDITY AND FATTY ACID COMPOSITION OF CELL MEMBRANES FROM THE SEA SCALLOP (*PLACOPECTEN MAGELLANICUS*) DURING SHORT-TERM COLD ACCLIMATION. J. M. Hall, R. J. Thompson, and **C. C. Parrish,** Ocean Sciences Centre, Memorial University of Newfoundland, St. John's, Newfoundland A1C 5S7, Canada.

Biological membranes are highly susceptible to the increases in membrane order and reduced membrane fluidity which result from a decrease in temperature. Ectotherms counteract these ordering effects of reduced temperature by adjusting the structural composition of the membrane, allowing thermal compensation of membrane function over wide ranges of environmental temperature, a process known as homeoviscous adaptation. The fatty acid composition of structural lipids is one of the most important factors controlling the physical state of biological membranes. In order to further our understanding of the role of PUFAs in cold ocean invertebrates, we incorporated a stearic acid electron spin label into the membranes of hemocytes and gill cells of sea scallops (*Placopecten magellanicus*) acclimated to 15C. The temperature in the scallop holding tanks was reduced to 5C over a 3 week period, during which cells were sampled at intervals. Membrane order (fluidity) was measured at 20C by electron spin resonance spectroscopy and the fatty acid composition of membrane phospholipids determined by gas chromatography. Phospholipid vesicles of cold acclimated scallops were more disordered (i.e. more fluid) than those of warm acclimated ones, and contained proportionately more PUFAs. The order parameter of the spin resonance signal was highly correlated ($r = -0.71$, $P < 0.001$) with the proportion of 20:5n-3, a PUFA which is generally believed to be important metabolically, yet no correlation was observed with 22:6n-3, a PUFA usually considered to have more of a structural function. The modulation of membrane phospholipid structure by 20:5n-3 (eicosapentaenoic acid, EPA) may be an important mechanism for thermal regulation of function in marine bivalves.

NATURAL SOURCES OF NUTRITION FOR THE MUSSEL *GEUKENSIA DEMISSA*. **Daniel A. Kreeger,*** Patrick Center for Environmental Research, Academy of Natural Sciences, Philadelphia, PA 19103; **Roger I. E. Newell** and **Shou-Chung Huang,** Horn Point Laboratory, University of Maryland, Cambridge, MD 21613.

Ribbed mussels (*Geukensia demissa*) are abundant in the intertidal zone of most eastern USA salt marshes where organic carbon inputs are apparently dominated by refractory detritus from angiosperms, such as *Spartina alterniflora*. To examine how ribbed mussels achieve such a high biomass where labile food resources may be limited, we integrated seasonal measurements of the availability of different constituents of natural seston with detailed physiological measurements of the mussel's ability to digest each component. Our analysis indicates that mussels are omnivores since no single food type can balance either their carbon or nitrogen demands on an annual basis. The major sources of carbon for *G. demissa* appear to be a mixture of phytoplankton and microheterotrophs (bacteria and bacterivorous flagellates), followed by smaller contributions from detrital cellulose and microphytobenthic diatoms and cyanobacteria. Phytoplankton are estimated to supply the bulk of the nitrogen demands of *G. demissa*, followed by a mixture of microheterotrophs and microphytobenthos. The relative ingestion, digestion and assimilation of these different food particles varies seasonally in proportion to their natural abundances, indicating that mussels feed optimally throughout the year. Importantly, the total bioavailable carbon from all of these foods exceeds the annual carbon demands of mussels; whereas, the nitrogen demands of *G. demissa* are not estimated to be met at any time of the year. This suggests that *G. demissa* may be nitrogen limited, and if so, nitrogen-rich foods are of paramount importance in the natural diet.

RIBOFLAVIN SUPPLEMENTS FOR LARVAL AND ADULT PACIFIC OYSTERS (*CRASSOSTREA GIGAS*) DELIVERED BY LIPID SPRAY BEADS. **C. J. Langdon,*** Hatfield Marine Science Center, Oregon State University, Newport, OR 97365, U.S.A., **C. Seguineau, B. Ponce, J. Moal,** and **J. F. Samain,** IFREMER, Laboratoire de Physiologie des Invertébrés, BP70, 29280 Plouzané, France.

Lipid spray beads (SB) were prepared containing 13%w/w particulate riboflavin. Beads agitated with seawater at 20–22 °C retained 27% riboflavin after 24 h of suspension. Oyster larvae were fed on riboflavin-SB and observed using an epifluorescent microscope. Riboflavin was released from ingested beads, causing the stomach contents of larvae to fluoresce green. Riboflavin concentrations in tissues of adult oysters fed on riboflavin-SB were significantly greater (SNK; $p < 0.05$) than those of oysters fed on seawater-filled SB. Significantly elevated (Students t-test; $p < 0.05$), sustained concentrations of riboflavin (11.2 μg/gDW) were observed in gonad tissue samples from broodstock supplemented with riboflavin-SB compared with those from non-supplemented controls (4.1 μg/gDW). There were no significant differences between riboflavin concentrations of eggs released from riboflavin-supplemented broodstock (21.7 μg/gDW) and those of controls (23.2 μg/gDW); however, egg hatching rates (73% versus 23%) and subsequent larval survival rates (90% versus 75% on day 7, and 82% versus 63% on day 23) were significantly higher (Students t-test, $p < 0.05$) for riboflavin-supplemented broodstock than for control broodstock.

PALLIAL CAVITY RESIDENCE TIME IN TWO SPECIES OF BIVALVED MOLLUSCS: *MYTILUS EDULIS* AND *CRASSOSTREA VIRGINICA*. **Lisa M. Milke*** and **J. Evan Ward,** Department of Marine Science, University of Connecticut, Groton, CT 06340.

Populations of bivalved molluscs can play a large ecological role by linking benthic and pelagic systems. Previous studies have shown that bivalves can compensate for changing food conditions through processes such as preferential selection and ingestion of particulate matter. Although the gross response of bivalves to changes in the quantity and quality of food have been examined, the underlying mechanisms responsible for these compensations are largely unknown. To address this issue, the fine scale feeding processes at the level of the ctenidia and labial palps were examined under conditions of differing particle quality. Pallial cavity residence time, or amount of time it takes a tracer particle to travel from the inhalent aperture to the stomach, was determined for *M. edulis* and *C. virginica*, and residence times on the labial palps were calculated.

Bivalves were offered one of three food types: *Rhodomonas lens* cells, particles prepared from ground *Spartina sp.* detritus, or a 50/50 mixture of both. Once actively feeding, bivalves were delivered 10 μm fluorescent polystyrene beads as a tracer. Bivalves were then removed at intervals from 30s up to 20 min and placed in liquid nitrogen, ensuring the cessation of particle transport. Digestive systems were isolated and examined for the presence of tracer beads, and pallial cavity residence times calculated. For mussels, it appears that food quality has little affect on pallial cavity residence time, as the residence time was ≤90s regardless of particle type. In oysters, tracer beads were initially detected at 30s when exposed to *R. lens* but not until 90s when feeding on the 50/50 mix. Pallial cavity residence time in oysters, when feeding on *R. lens* and the 50/50 mix, was twice as long as in mussels, perhaps due to extensive particle processing on the ctenidia. Furthermore, oysters given *Spartina sp.* particles were still lacking the presence of beads in their gut even after 20 minutes of feeding. These results suggest that food quality and degree of particle processing on the ctenidia and labial palps may affect feeding rate.

PARTICLE FLUX AND CONSUMPTION BY MUSSELS AT ROQUE ISLAND, MAINE: THE IMPORTANCE OF MARINE SNOW. **Carter R. Newell,** Great Eastern Mussel Farms, Tenants Harbor, ME 04860; **Cynthia Pilskaln,** School of Marine Sciences, University of Maine, Orono, ME 04469; **Shawn Robinson,** St. Andrews Biological Station, Department of Fisheries and Oceans, St. Andrews, New Brunswick, Canada EOG2XO; **Bruce MacDonald,** Department of Biology, University of New Brunswick at St. John, St. John, New Brunswick, Canada E2L4L5.

During three days in July of 1998, the flux and consumption of seston by mussels, *Mytilus edulis,* was studied at a low-current mussel bottom lease in Englishman's Bay, Maine. The experimental approach involved feeding studies in flow-through chambers, benthic video of mussels on the bottom, periodic water grab samples, nightly profiles of marine snow concentration, current measurements and sediment traps deployed over 24 hours to collect the settling flux of particles. Mussels feeding on surface water produced no pseudofeces, in contrast to the bottom mussels where the rates were positively correlated with exhalant siphon area of the mussels. The volume of marine snow increased with water depth, resulting in a pulse of material, largely inorganic, to the mussels on bottom on the ebb tide. The food supply of mussels due to settling and vertical mixing, with respect to particulate carbon and nitrogen was similar, but with respect to inorganic sediments was nearly twice as high for the settling flux than for vertical mixing. Therefore, marine snow, at least in mid-summer during our study, had a negative effect on mussel growth in bottom culture.

FOOD QUALITY AND FEEDING STRATEGIES IN HATCHERY REARING OF PACIFIC OYSTER *CRASSOSTREA GIGAS* LARVAE; A MODELING APPROACH. **Eric Powell,*** Haskin Shellfish Lab., Rutgers Univ., Port Norris, NJ 08349; **John Klinck** and **Eileen Hofmann,** CCPO, Old Dominion Univ., Norfolk, VA 23529; **Eleanor Bochenek,** N.J. Sea Grant College Program, Sandy Hook Field Station, Fort Hancock, NJ 07732.

A biochemically-based model was used to simulate the growth and metamorphosis of hatchery-reared *Crassostrea gigas* larvae. The model includes parameterizations of the metabolic costs associated with larval filtration, ingestion, and respiration. The initial biochemical content of the larva is determined by egg composition. Changes in the ratios of protein, carbohydrate, neutral lipid and polar lipid occur as the larva grows in response to environmental conditions. Model simulations show increased larval survival when low-protein diets are provided. High-protein diets do not provide the lipid resources necessary to maintain the optimal protein to polar lipid ratio in the larva's structural components while still providing sufficient energy for metamorphosis. Thus, food quality is an important factor controlling the ability of *C. gigas* larvae to survive and metamorphose. Additional simulations show that small (a few hours) variations in daily food supply, such as daily or twice-daily feeding, cause large changes in survival rate. This may provide one explanation for widely varying survival rates under hatchery conditions. Simulations also show that larger eggs with greater lipid content produce larvae that are more able to withstand poor food environments over the larva's life, suggesting that factors associated with brood stock conditioning, as they influence egg quality, may significantly influence hatchery survival of spawn. However, eggs can be too large. Optimal size, around 50 μm, yields greatest metamorphosis success. Many of these observations support longstanding practices in successful oyster hatcheries. The model provides one way to examine optimization schemes in hatcheries without detailed and expensive experimentation.

THE NUTRITIONAL VALUE OF *PALMARIA MOLLIS* CULTURED UNDER DIFFERENT LIGHT INTENSITIES AND WATER EXCHANGE RATES FOR JUVENILE RED ABALONE *HALIOTIS RUFESCENS*. **Gunther Rosen, Chris J. Langdon,** and **Ford Evans,*** Hatfield Marine Science Center, Oregon State University, Newport, OR 97365.

The co-culture of red abalone (*Haliotis rufescens*) and the red macroalgae, dulse (*Palmaria mollis*), has been shown to be an effective method of abalone production. In this study we examined the effect of dulse culture conditions on it's nutritional quality for juvenile red abalone. Culture conditions differed by seawater volume exchange rate (1, 6, or 35 d^{-1}) and presence or absence of supplemental illumination (0 or 24 h d^{-1}), creating a variety of dulse types that differed in morphology and biochemical composition. The results showed dulse to be of high nutritional quality for abalone. Specific growth rates (SGR) of abalone fed on all dulse diets were higher than those of abalone fed on kelp (*Nereocystis luetkeana*), the macroalgal diet commonly used for abalone culture in the United States. Protein content of dulse (10.85 to 18.22% dry wt.) generally increased with increasing seawater volume exchange rate. Abalone growth rate, however, was primarily affected by light supplementation (ANOVA; $P < 0.01$) and not water volume exchange rate (ANOVA; $P > 0.05$). Therefore, other variables apart from protein content, such as the abundance of epiphytic diatoms and morphological differences of thalli, may have contributed to higher growth rates of abalone fed on light-supplemented diets.

GIGAS, NUTRITION AND GAMETOGENESIS: PRESENTATION AND FIRST RESULTS OF THE EUROPEAN PROJECT GIGANUGA. **J. F. Samain,* C. Quere, J. R. Le Coz, C. Seguineau, P. Soudant,** and **J. Moal,** Laboratoire de physiologie des invertébrés, IFREMER centre de Brest, BP 70, 29280 Plouzané, France; **P. Sorgeloos, M. Caers,** and **C. Van Ryckeghem,** Laboratory of Aquaculture & Artemia reference center, University of Gent, Rozier 44, 9000 Gent, Belgium; **O. Garcia** and **J. Espinosa,** Department of Biochemistry and Molecular Biology, University of Santiago de Compostela, 15706 Santiago de Compostela, España; **Y. Marty,** UMR/CNRS 6521, Université de Bretagne Occidentale, BP 809, 29285 Brest, France; **M. Mathieu** and **C. Berthelin,** Laboratoire de Biologie et de Biotechnologies Marines, Université de Caen, IBBA, 14032 Caen, France.

This European project aims at the improvement of broodstock management in oyster hatcheries through a better understanding of the relation between broodstock nutrition and quality of early life stages, with the following specific objectives: 1—Document problems in current hatchery practice by comparison of nutritional aspects of reproduction under natural and artificial conditioning. 2—Identify critical nutrients for broodstock nutrition and define artificial diets to supplement these nutrients to live algae, taking into account the initial nutrient storage of broodstock. 3—Improve cost-efficiency of broodstock conditioning.

Five aspects of the very first results will be reported: 1/—Keypoints of the cellular aspects during the reproductive cycle of C. gigas in nature and in hatchery. 2/—What is the biochemistry of a normal reproductive cycle today? Biochemical aspects of the reproductive cycle in nature and in hatchery: glycogen, essential fatty acid, sterol or vitamin (somatic, germinal and egg compartments). 3/—Supplementation methodologies, efficiency in liposoluble or hydrosoluble molecule transfer of the different artificial particles tested. 4/—Effect of artificial supplementations during conditioning of C. gigas fed a standard algal mixture and a low cost algal diet (spring experiments, histology, biochemistry and reproduction performances) progress and questions. 5/—Reproduction at fall, a combination of physical and nutritional factors perspectives for a better reproduction process in hatcheries.

LIPIDS REQUIREMENTS IN SOME ECONOMICALLY IMPORTANT MARINE BIVALVES. **Philippe Soudant*** and **Fu-Lin E. Chu,** Virginia Institute of Marine Science, College of William and Mary, Gloucester Point, VA 23062, USA; **Jean-Francois Samain,** DRV/A, Laboratoire de physiologie des mollusques, IFREMER centre de Brest, BP 70, 29280 Plouzané, France.

Lipids play an important role in bivalves' reproduction, development and growth. Phytoplankton, the primary food source for bivalves, provides essential sterols and polyunsaturated fatty acids (PUFAs), such as 22:6n-3 (DHA), 20:5n-3 (EPA), and 20:4n-6 (AA), although the amount of these components vary with species and seasons. Dietary DHA, EPA, AA and sterols are critical for most bivalves' growth and reproduction. Generally, the ability to synthesize the above PUFAs and sterols in bivalves are limited. PUFA and sterol contents in wild oysters (e.g., *Crassostrea virginica* and *C. gigas*) and scallops (*Pecten maximus*) have been found to be associated with their diets and reproductive cycle. Sterol and PUFA composition of microalgae used in a hatchery-nurseries significantly influenced the fatty acid and sterol composition of the reared larvae, spat and broodstock of *C. virginica, C. gigas,* and *P. maximus.* Results of studies focused on the phospholipid fatty acid and sterol compositions in *P. maximus, C. virginica,* and *C. gigas* revealed a selective incorporation of PUFAs and cholesterol in structural lipids. DHA is an essential structural component, assimilated specifically in some phospholipid classes. Its dietary deficiency is related to gametogenic, embryogenic, and metamorphosis impairments in *P. maximus.* EPA is considered to be an energetic rather than structural component during embryogenesis and larval growth in *P. maximus.* Like most organisms, AA in scallops and oysters preferentially resides in phosphatidylinositol and is believed to be involved in cellular signaling. However, the precise functions of specific PUFAs and their conservation in phospholipid classes remains to be elucidated. Although the qualitative lipid requirements in most economically important species are generally known, their quantitative requirements are unclear. The nutritional values of various cultured algal species, for several economically important bivalves, will be reviewed and discussed according to their lipid composition.

ALGAL UPTAKE RATE OF FRESHWATER MUSSELS. **Kevin R. Stuart*** and **Arnold G. Eversole,** Department of Aquaculture, Fisheries and Wildlife, Clemson University, Clemson, SC 29634; **David E. Brune,** Department of Agriculture and Biological Engineering, Clemson University, Clemson, SC 29634.

Algal rich water was provided at nine flow rates in six temperature ranges to *Elliptio complanata.* Algal uptake rates were measured using particulate organic C/kg of wet mussel tissue/hr at 4 hr intervals from 0700 to 1900 over a 72-hr period. The water contained 12 algal taxa dominated by *Scenedesmus* (82%), *Merismopedia* (7%), and *Ankistrodesmus* (3%). Mussel uptake rates increased initially after being placed in the filtering chambers before stabilizing at a rate specific for each flow rate. These mean (± SD) uptake rates were 35.5 ± 3.54, 42.2 ± 7.16, 67.9 ± 7.34, 141.8 ± 16.2, 183.0 ± 13.19, 254.6 ± 46.81, 284.6 ± 27.33, 309.4 ± 13.99, and 311.2 ± 8.92 mg C/kg/hr at 0.07, 0.1, 0.2, 0.6, 1.0, 1.5, 2.0, 2.5, and 3.0 L/min of water at 27.4 ± 0.5 °C, respectively. Uptake rates increased as water temperatures increased from <10° and 10–15 °C to 15–25 °C reaching a maximum uptake rate at 25–30 °C. Uptake rates at water temperatures >30 °C were reduced to levels lower than that observed at 15–20 °C. Uptake rates increased as all concentrations (mgC/L) increased until reaching an asymptotic level distinct for each water temperature range. *E. com-*

planata filtered approximately 4.8% to 24.5% of their tissue wet weight in wet weight of algae daily. Comparison with other mussels species indicate species specific uptake rates.

CHANGES IN THE FATTY ACID COMPOSITION OF THE FLAGELLATE *PAVLOVA PINGUIS* (CCMP459) DURING CULTURE. **S. C. Feindel, R. J. Thompson,** and **C. C. Parrish,** Ocean Sciences Centre, Memorial University of Newfoundland, St. John's, Newfoundland A1C 5S7, Canada.

Our previous work, reported at the 1999 meeting of NSA, showed that larvae of the sea scallop *Placopecten magellanicus* grew more rapidly on a diet of *Pavlova* sp. (CCMP459) than on other unialgal diets examined. In addition to possessing a balanced distribution of n-3 fatty acids, CCMP459 was unusually rich in n-6 PUFAs, especially 20:4n-6 and 22:5n-6. The observed effectiveness of CCMP459 in supporting growth of the larvae of a cold water bivalve may be associated with the generally high incidence of PUFAs in the food chain in cold oceans and their importance in maintaining membrane fluidity at low temperatures (unpublished observations). We have now extended this work by examining the lipid content and fatty acid composition of CCMP459 in more detail at various phases of culture. A cell in stationary phase contained twice as much lipid as one in exponential phase, a property common to many algal species in culture. A broader array of fatty acids was observed in CCMP459 than is seen in most food species used in bivalve hatcheries, and although significant levels of 22:5n-6 have been recorded in other strains of *Pavlova*, the richness of CCMP459 in 20:4n-6 appears to be unusual. As the culture aged and became more nutrient and light limited, levels of n-6 PUFAs increased at the expense of 20:5n-3 and, to a lesser degree, 22:6n-3. Saturated FAs were not substantially elevated during the stationary phase, and PUFA content was high during all growth phases, which is inconsistent with many published studies on a variety of algal cultures. Further work is required to determine the role of 22:5n-6 in the biology and culture of the sea scallop and other cold water bivalves.

INFLUENCE OF DIET QUALITY ON PRE-INGESTIVE FEEDING STRATEGIES OF BIVALVES: CONNECTING PALLIAL CAVITY FUNCTION TO ECOSYSTEM PROCESSES. **J. Evan Ward,*** Department of Marine Sciences, University of Connecticut, Groton, CT 06340; **Jeffrey S. Levinton,** Department of Ecology & Evolution, S.U.N.Y., Stony Brook, NY 11794; **Sandra E. Shumway,** Natural Science Division, Southampton College, Southampton, NY 11968; **Terri L. Cucci,** Bigelow Laboratory for Ocean Sciences, Boothbay Harbor, ME 04575.

The compensatory responses of bivalved molluscs to changing diet quantity and quality have been examined for a number of species. Previous studies suggest that the ability of bivalves to adjust ingestion rates and reject non-nutritive particles as pseudofeces, for example, is critical to their survival. The mechanisms that underlie these observed feeding compensations, however, have not been well studied. We hypothesize that fine-scale adjustments at the level of the gill and labial palps are what ultimately define the integrated response of the individual.

To examine some of these fine-scale adjustments, we exposed oysters (*Crassostrea virginica* and *C. gigas*) and mussels (*M. edulis, M. trossulus*) to a mixture of ground, aged *Spartina* sp. (3–10 μm) and similar sized phytoplankton (*Rhodomonas* sp.) at three concentrations (10^3, 10^4, 10^5 particles ml^{-1}). We then observed the gills and labial palps of bivalves by means of video endoscopy, examining aspects of particle handling and transport, and collecting discrete samples from pallial organs. We also performed a number of particle depletion and residence time experiments to measure time course of particle handling by gills and labial palps. Our results indicate that changes in diet quality do affect pallial organ processes, including the route (dorsal vs. ventral tracts) and rate at which particles are transported to the labial palps, the residence time of particles on the gills and labial palps, and particle rejection. Gut fullness mediates pallial cavity processes, and the magnitude and type of response elicited differs between oysters and mussels. Our data suggest that fine-scale adjustments in pallial cavity processes are the underlying mechanisms of previously observed compensatory responses to changing diet qualities. Studies such as these will lead to a better understanding of pallial organ function, and allow us to better model the critical limiting factors that mediate particle-feeding in bivalves and ultimately affect the trophic dynamics of benthic ecosystems.

AQUACULTURAL FEEDING STANDARDS FOR MOLLUSCAN SHELLFISH SEED: A FIRST CUT. **Gary H. Wikfors,*** Milford Laboratory, Northeast Fisheries Science Center, NOAA Fisheries, Milford, CT 06460.

Domestication of animals for human food requires an understanding of the nutritional needs of the animals. Nutritional needs include aspects that are both qualitative (what) and quantitative (how much and how often). Decades of research in animal agriculture have led to the establishment of feeding standards for common livestock (cattle, poultry, swine, sheep) that list daily energy and biochemical inputs necessary for desired growth (or other performance characteristics), based upon the size and maturity of an individual animal. In the US, these feeding standards are published, and updated periodically, by the National Research Council (NRC) and are used throughout the agriculture industry in formulating commercial feeds and supplements. Domestication of molluscan shellfish can benefit from application of the feeding stan-

dards concept, particularly for younger stages that are reared in land-based systems wherein control over nutritional input is possible.

Research in the Milford Laboratory has focused upon the "what," "how much," and "how often" of molluscan nutritional needs from the perspective of controlled aquaculture, rather than descriptive ecology. Experiments comparing many algal strains as feeds for juveniles of both eastern oysters, Crassostrea virginica, and bay scallops, Argopecten irradians, have identified several, high-lipid strains in the algal genus Tetraselmis as being the most-nearly complete nutritionally, on a qualitative basis. Subsequent experiments, employing a computer-controlled feeding apparatus, sought to optimize quantitative aspects of the delivery of Tetraselmis diets to both oysters and scallops. Data from these experiments, and from biochemical analyses of the Tetraselmis strains, have been used to calculate provisional feeding standards for juvenile oysters and bay scallops in a format similar to that of the NRC agricultural feeding standards. These provisional feeding standards for juvenile oysters and scallops will be presented and compared, where possible, with those developed in agriculture, and needs for subsequent research will be identified. Development of practical feeding standards will benefit molluscan aquaculture by providing a framework within which both live and prepared dietary components may be incorporated.

FORUM: BIOLOGICAL AND TECHNOLOGICAL TRANSFERS IN SHELLFISH

BIOLOGICAL AND TECHNOLOGICAL TRANSFERS IN SHELLFISH AQUACULTURE. Joth Davis, Baywater Inc. 15425 Smoland Lane, Bainbridge Island, WA 98110, USA.

Technology transfer efforts from research to industry in aquaculture have followed a similar pathway as in other agricultural industries in that the route often involves the need to protect intellectual property. The result is that the dissemination of information and the timely utilization of technological innovations may become constrained and less readily adapted by industry. Transfers of technology may involve a small component or process which may help to streamline or economize an operation, a genetic technique or process which produces fundamentally different plants or animals with enhanced value for culture, or wholesale transfers or introductions of information including technology to parts of the world where shellfish culture is constrained or otherwise underdeveloped.

Too often, transfers from academia to industry have been constrained due to the actual laws or regulations, but also to ethical considerations concerning the protections afforded intellectual property. This forum seeks to bring together a group of practitioners from academia and industry, and within the legal field who have had experience in a variety of technological transfers within the field of shellfish biology and aquaculture. In this forum we hope to provide information and generate discussion about the process, the pitfalls and the opportunities for streamlining technology transfers specific to shellfish culture for the future.

FORUM: THE APPROPRIATENESS OF CULTURING BIVALVES FOR FOOD, PROFIT, RESOURCE RESTORATION, HABITAT AND WATER QUALITY MITIGATION

FORUM ON THE APPROPRIATENESS OF CULTURING BIVALVES FOR FOOD, PROFIT, RESOURCE RESTORATION, HABITAT AND WATER QUALITY MITIGATION. William F. Dewey,* Taylor Shellfish Farms, S.E. 130 Lynch Road, Shelton, WA 98584; **Daniel P. Cheney,*** Pacific Shellfish Institute, 120 State Avenue NE #142, Olympia, WA 98501.

This forum addresses the question: Is there a role for bivalve culture in estuarine systems for food, profit, resource restoration, habitat and water quality mitigation? As we enter the 21st century the future of shellfish culture in estuaries around the world could be described as very bleak or ultimately promising. If one is trying to produce safe, wholesome shellfish on a farm for profit, along urbanizing shorelines, with declining water quality, endangered species, demands for pristine views and increased spatial demands by competing users, the future looks bleak. On the other hand, if one is attempting to restore oysters as a keystone species to the Chesapeake Bay or New York Harbor to help consume excess algae and create reef structures and fish habitat or reestablish the Native Olympia oyster in Puget Sound the future looks intriguing. With recent trends towards ecosystem management a greater diversity of individuals with varying professional backgrounds and opinions are involved in making resource management decisions. Support for shellfish culture varies dramatically and depends on the region of the country and/or the backgrounds of the individuals influencing local resource management decisions. This forum is intended to facilitate a dialogue that explores the pros and cons of culturing shellfish for food, profit, resource restoration, habitat and water quality mitigation, and address the nature of the varying positions and attitudes regarding the role of bivalve culture in estuaries.

FUNCTIONAL ROLE OF BIVALVES IN MARINE ENVIRONMENTS

OYSTER REEFS AS ESSENTIAL FISH HABITAT FOR FINFISH AND DECAPOD CRUSTACEANS: A COMPARISON FROM NATURAL AND DEVELOPING REEFS.

Loren D. Coen,* Marine Resources Research Institute, South Carolina Department of Natural Resources, Charleston, SC 29412; **Mark W. Luckenbach,** VIMS, Eastern Shore Lab, P.O. Box 350, Wachapreague, VA 23480; **Denise Breitburg,** The Academy of Natural Sciences, Estuarine Research Center, 10545 Mackall Rd., St. Leonard, MD 20685.

Until recently our knowledge base on the value of oyster-dominated habitats for the maintenance of economically- and ecologically-important species was extremely limited, especially relative to other biogenic habitats such as seagrasses, mangroves or saltmarsh. In most cases the assigned value of shellfish habitats and their conservation/protection was based exclusively on resource value, accessibility and public health, but of late this has begun to change. Here we summarize the current status of oyster reefs as 'Essential Fish Habitats' (or EFH) by: (1) first making a case for broadening our understanding of the ecological functions of shellfish habitats; (2) summarizing the current state of our knowledge on oyster habitat utilization patterns by both resident and transient finfish and decapod crustaceans from the Chesapeake Bay to the Gulf of Mexico; (3) discussing how current restoration projects are enhancing our perspective; and finally (4) emphasizing the necessity of an integrated ecosystem (or adaptive) management approach regarding their management and restoration. We also make recommendations for future EFH efforts.

BIVALVES OR NEKTON? IS THAT THE QUESTION?

Richard Dame,* **David Bushek, Dennis Allen, Alan Lewitus, Eric Koepfler, Leah Gregory,** and **Don Edwards,** Baruch Marine Field Laboratory and Department of Statistics, University of South Carolina, Georgetown, SC 29442 and Coastal Carolina University, Conway, SC 29528.

An ongoing ecosystem scale experiment in which oysters are completely removed from tidal creeks is described and used as a case study. The experimental design takes estimates of the system's carrying capacity into account. Using the population or species approach to monitor the oysters, the only observable change after the experimental manipulation was a slight increase in summer somatic growth and elevated recruitment of oysters in creeks with oyster reefs removed. These data are interpreted as an indication that the creeks with oysters present are below or near carrying capacity. However, when nekton, plankton and water chemistry data are also examined a much more complicated picture emerges. During the summer growing season, nekton biomass in all creeks is often greater than oyster biomass. Also, our calculations show that oysters do not produce enough ammonium to satisfy phytoplankton productivity, but nekton, water column remineralization and sediments can account for most of the deficit. Finally, nanoflagellates, which are a preferred food for the oysters, dominate the phytoplankton during the summer growing season and diatoms dominate the colder months. The change in phase of phytoplankton dominance coincides with the seasonal arrival and departure of nekton in the creeks.

We argue that dense bivalve reefs and beds are indicative of intense positive feedback loops that make their ecosystems fragile and susceptible to dramatic changes in structure. Such changes have not been reported for natural systems, but are found in systems influenced by over-fishing, nutrient loading and pollution. Thus, the management of sustainable fisheries in coastal ecosystems requires an understanding of the ecosystem science and the realization that tidal creek systems exhibit complex responses that are not easily explained by linear dynamics.

OYSTER AQUACULTURE AND BENTHIC INVERTEBRATE COMMUNITIES IN WEST COAST ESTUARIES: AN UPDATE.

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A review of a limited number of field studies suggests that oyster aquaculture practices play a key role in structuring the benthic macro-invertebrate community in west coast estuaries. Oysters are "bioengineers" as they change the structure of the substrate and create habitat for other organisms. Macro-invertebrate communities are typically enhanced in intertidal ground culture oyster habitat as compared to other estuarine habitats, in particular intertidal mud and burrowing thalassinid shrimp dominated habitats. Oysters add structure for macro-algal, mussel and barnacle attachment which in turn provide protection and/or food for juvenile Dungeness crab, shore crabs *Hemigrapsus*, tube building gammarid amphipods such as *Amphithoe* and *Corophium*, caprellid amphipods, tanaids, and some annelids such as the scaleworm *Harmothoe*. Two other bioengineers, the ghost shrimp *Neotrypaea californiensis* and the mud shrimp *Upogebia pugettensis*, dominate large portions of the intertidal in some west coast estuaries and compete for space with oysters. These thalassinid shrimp create a soft, highly burrowed habitat suitable for other burrowing organisms like the amphipods *Eohaustorius* and *Eobrolgus*, the polychaete *Mediomastus*, and some commensal organisms like the clam *Cryptomya*, but support fewer filter feeders and much lower species diversity than oyster habitat. Preliminary results of a recent study in Willapa Bay, WA comparing the macro-infaunal community in ground culture oyster habitat with that in six other estuarine habitats are presented along with a review of previous studies in

West coast estuaries. To date, little has been done to estimate functional effects of these changes at the larger estuarine ecosystem scale, but some proposed work aims to investigate the functional role of these habitats for the estuarine fish community.

JUVENILE OYSTER GROWTH AND CARRYING CAPACITY OF INTERTIDAL CREEKS IN NORTH INLET, SC. **A. J. Erskine*** and **David Bushek,** Baruch Marine Field Laboratory, University of South Carolina, Georgetown, SC 29442; **Richard Dame,** Department of Marine Science, Coastal Carolina University, Conway, SC 29528; **Nancy Hadley** and **Loren Coen,** Marine Resources Research Institute, South Carolina Department of Natural Resources, Charleston, SC 29412.

In North Inlet Estuary, South Carolina, natural populations of eastern oysters grow in dense assemblages that form extensive intertidal beds and reefs. High oyster recruitment leads to intense fouling of hard structures placed in the intertidal zone. These observations indicate a highly productive system. The density of oysters in small (100–400 m long) intertidal creeks ranges from 2.3 to 27.5 g dry body wt m^{-3} (or about 7 to 100 adult oysters m^{-3}) with a mean of 10 g and median of 6.3 g. Based on this information, the density of oysters in eight intertidal creeks was adjusted to 8 g dry body wt m^{-3} as part of an NSF-funded study (designated 'CREEK' Study) to determine the overall ecological role of oysters in tidal creek ecosystems. We hypothesized that this density was near the average carrying capacity of oysters for these tidal creeks. To test this hypothesis, we examined the growth of juvenile SPF-oysters deployed in cages in the eight intertidal creeks, before and after the removal of native oysters from four of the creeks. Prior to removal of native oysters there was little difference in juvenile oyster growth rates deployed in the creeks. Following removal of native oysters, deployed oysters grew faster in removal creeks vs. those deployed in control creeks (oysters present at 8 g dry body wt m^{-3}). These data indicate that oysters in these small tidal creeks may be at or near the carrying capacity for the system.

NATURAL INTERTIDAL OYSTER REEFS IN FLORIDA: CAN THEY TEACH US ANYTHING ABOUT CONSTRUCTED/RESTORED REEFS? **Ray Grizzle,** Jackson Estuarine Laboratory, University of New Hampshire, Durham, NH 03824; **Mike Castagna,** Virginia Institute of Marine Science, Eastern Shore Laboratory, Wachapreague, VA 23480-0350.

Distribution and abundance patterns at various spatial scales of the natural, intertidal oyster reefs in the Canaveral National Seashore, Florida may be instructive with respect to the design of constructed/restored reefs, whether intertidal or subtidal. Areawide reef patterns were characterized using low-altitude aerial imagery and GIS-based mapping. Inter- and Intra-reef patterns in oyster size and abundance were characterized using quadrat sampling on 10 reefs. Lagoon-wide there was a strong south-to-north increase in areal coverages by the reefs correlated with increasing tidal ranges. Tide range was also positively correlated with adult and spat densities, but not oyster size. Although a quantitative analysis has not been done, localized, inter-reef patterns showed a strong relation to tidal flows. The largest reefs and many smaller reefs were oriented parallel to and/or along the edges of major tidal channels, as commonly reported in other areas. In some areas, however, there were clusters of reefs arranged in dendritic patterns associated with multiple tidal channels. Theoretical models indicate that such a pattern can cause greater mixing of the water column and thus of food transport compared to single reefs of similar total size. Intra-reef patterns included an "edge effect" on some reefs with much greater spat settlement and oyster densities within a 2 to 3 m fringe. There was no clear relationship, however, between intra-reef spatial variations and reef size or location. Patterns on all three spatial scales indicate that water movements are of major importance to reef development and maintenance. They also reflect the complexities involved in cause-and-effect relationships and may provide insight into the design of constructed reefs.

THE ECOLOGICAL IMPLICATIONS OF HIGH DENSITY HARD CLAM (*MERCENARIA MERCENARIA*) MARICULTURE ON TIDAL CREEK ENVIRONMENTS. **Michael L. Judge,*** Department of Biology, Manhattan College, Riverdale, NY 10471; **Loren D. Coen,** Marine Resources Research Institute, SCDNR, Charleston, SC 29412; **Kamille Hammerstrom,** NOS, Beaufort Lab, Beaufort, NC 28516.

Hard clams, common along the Atlantic U.S. coast and northern Gulf of Mexico, have historically supported a valuable fishery. Although wildstock landings have remained constant or decreased over the last decade, mariculture production has increased steadily, with several large clam aquaculture operations established in VA, NC, SC and FL. The deployment of hundreds to thousands of clam culture pens, each with tens of thousands of clams has the potential to affect: (1) local hydrodynamics, (2) sediment characteristics, (3) associated benthos, (4) food quality and quantity and, (5) ultimately the carrying capacity of the local habitat. The consequences of the above can have both direct and indirect impacts for both the mariculture industry and the environment. In SC, hard clam culture is performed on low intertidal mudflats within tidal creeks that are typically surrounded by dense oyster reefs (*Crassostrea virginica*). In 1997, we initiated a study, in conjunction with a large clam aquaculture facility to address in part the above concerns within an intertidal soft-bottom system typical of southeastern U.S. The structural presence of cages imparted profound changes in the hydrodynamic regimes within and around clam pens, thereby altering numerous sediment attributes (such as, grain size, chlorophyll *a* concentrations, and C/N ratios). Moreover, the localized de-coupling of the benthic boundary layer owing to cage-induced mixing dramatically affected the temporal variation of resuspended algal food supplies. Over longer time scales, the pens

themselves provide additional hard substrate habitat for non-target species (oysters, sponges, tunicates). The maintenance and ultimate removal of caging materials present additional future concerns.

TEMPORAL PATTERNS OF FISH AND DECAPOD UTILIZATION OF OYSTER REEFS: COMPARISONS ACROSS AN ESTUARINE GRADIENT. **Mark Luckenbach*** and **Francis O'Beirn,** Virginia Institute of Marine Science, Eastern Shore Lab, College of William and Mary, Wachapreague VA 23480; **Juliana Harding, Roger Mann,** and **Janet Nestlerode,** Virginia Institute of Marine Science, College of William and Mary, Gloucester Point, VA 23062.

Biogenic reefs created by the eastern oyster *Crassostrea virginica* are increasingly being recognized for their associated biodiversity. Yet, few details of the use of oyster reefs as habitat for mobile species of fish and decapods are available. Specifically, the temporal patterns of species use of reefs and the nature of that use (e.g., refugia or foraging) are largely unknown. At two restored reef sites in the Chesapeake Bay, one in a polyhaline and one in a mesohaline environment, we have conducted multi-year studies of the development of reef communities and their utilization by mobile fauna. Here we report on variation in use of these reef habitats by finfish and crabs on several temporal scales: seasonal, fortnightly, diel and tidal. Using fixed and towed nets and underwater video, we compare the abundances of fish and crabs on the reef with that of adjacent unstructured habitats. Direct observations of feeding activity and gut analysis are used to clarify trophic linkages. Our findings indicate that oysters and the resident assemblages which they support may play a significant role in supporting several commercially and recreationally important species.

GRAZING OF NATURAL PARTICULATES BY BLUE MUSSELS ON RAFTS: SIMULATIONS USING FLOW-3D. **Carter R. Newell,*** Great Eastern Mussel Farms, Tenants Harbor, ME 04860; **John E. Richardson,** Earth Tech, Concord, NH 03301.

Since the early 1980's blue mussels have been cultivated on the bottom in Maine, and more recently mussel culture on rafts has become popular on both the west and east coasts of the U.S. The supply and demand of particulate food to mussels on the bottom has been previously quantified in a model MUSMOD, which has recently been improved to include the settling flux of particles as well as those supplied to the bottom by advection and vertical mixing. On rafts, the effects of the mussel lines and predator nets on current speed result in a complex pattern of flow around and through the rafts, which we have successfully simulated using FLOW-3D. Food availability within and around the rafts was measured by water grab samples and using a CTD with a fluorometer. Depletion of food particles by as much as 50% were measured in the field, and simulated in a model relating particle consumption by the mussels to their biomass on the ropes. Initial model runs were then used to optimize mussel growth rates and yield by changing raft orientation to flow, rope spacing and seed density. Sedimentation rates of mass, carbon and nitrogen were also measured and compared with control stations.

Adequate currents are required to provide food to the middle of mussel rafts, and also to provide oxygen to benthic bioturbators feeding on the organic matter accumulation below the rafts.

ROLE OF SUSPENSION FEEDING BIVALVES IN MEDIATING ESTUARINE NUTRIENT CYCLING. **Roger I. E. Newell*** and **Jeff C. Cornwell,** Horn Point Laboratory, UMCES, PO Box 775, Cambridge, MD 21631, USA.

Although it is apparent that changes in the abundance of benthic suspension feeding bivalve molluscs will proportionally alter the degree of benthic pelagic-coupling the ensuing ecosystem changes are less predictable. That is, will an increase in bivalves just result in faster inorganic nutrient recycling, thereby stimulating phytoplankton production, or will the rate of nutrient recycling be different than if the same amount of organic material was degraded in the water column? In laboratory mesocosms under oxic and anoxic conditions in the dark we measured changes in sediment geochemistry, nutrient fluxes, and denitrification in response to loading by different amounts of algal paste, an experimental analog of oyster biodeposits. Increased organic loading to the sediment under oxidized conditions resulted in higher rates of coupled nitrification/denitrification. In contrast, coupled nitrification/denitrification was suppressed under anoxic conditions. Similar incubations in the light which permitted the growth of benthic microalgae showed negligible ammonium fluxes from sediments, with the algal/microbial community efficiently retaining ammonium and fixing nitrogen. Because no DIN was recycled to the water column under oxic conditions we conclude that increasing the stocks of suspension feeders stocks will have the beneficial effect of removing phytoplankton from the water column without stimulating further phytoplankton production. Furthermore, net rates of nitrogen loss via denitrification will be enhanced in areas with higher levels of benthic-pelagic coupling.

INTERRELATIONSHIPS BETWEEN SEAGRASSES AND BENTHIC SUSPENSION FEEDERS. **Bradley J. Peterson,*** Department of Biological Sciences, Florida International University, Miami, FL 33199; **Kenneth L. Heck, Jr.,** Dauphin Island Sea Lab, Dauphin Island, AL 36528.

Two simultaneously conducted field experiments using live mussel density manipulations and a 3 × 3 factorial incomplete randomized design utilizing mussel mimics and nutrient enrichment of the sediments were conducted to examine the effect of mussels on meadows of the seagrass *Thalassia testudinum*.

The live mussel density manipulations resulted in significantly increased nutrient concentration of sediment porewaters, significantly reduced leaf tissue C:N, N:P and C:P ratios and reduced epiphytic loads, and increased seagrass production when mussels were present. The 3 × 3 factorial design tested the separate factors of increased habitat structure and increased nutrient enrichment resulting from the presence of the mussels. Structure had a significantly negative effect on epiphytic biomass. Nutrient had a significantly positive effect on sediment porewater nutrient concentrations and a significantly negative effect on leaf tissue N:P and C:P ratios. The strength of the positive response to mussel presence in seagrass productivity appeared to progress along a continuum from early reliance on nutrient enrichment to the increasing role of habitat complexity as the growing season advanced.

Finally, a field experiment evaluating the effects of seagrass on the survivorship of the associated mussel, *Modiolus americanus* was conducted. Mean survival was significantly greater in vegetated habitats than in unvegetated sediments. Thus, this study demonstrates the reciprocal positive interactions of these organisms when associated and suggests that seagrass meadows may exist as a mosaic of patches of differing productivity when suspension feeding organisms are present.

USE OF OYSTER REEFS BY MOBILE FAUNA: CONSEQUENCES FOR ADJACENT SANDFLAT HABITATS. **Martin H. Posey** and **Troy D. Alphin**, Depart. Biological Sciences, UNC-Wilmington, Wilmington, NC 28403; **Christopher M. Powell**, Center for Marine Science Research, UNC-Wilmington, Wilmington, NC 28403; **John M. Rhoads**, Barry A. Vittor & Assoc., 271 Zena Rd., Kingston, NY 12401.

There has been increasing recognition of the importance of oyster reefs as habitat for benthic fauna and nekton. However, the importance of landscape parameters in the function of oyster reefs is less well understood. Among these landscape considerations are the potential interactions between organisms inhabiting oyster reef communities and those in adjacent habitats. Oyster reefs provide refuge for a variety of resident predators, such as rock crabs, gobies and certain shrimp and transient predators such as blue crabs and pinfish. Research on coral reefs, hardbottom outcrops, and artificial reefs indicates that reef-associated predators often increase predation intensity on adjacent sandflat habitats, suggesting the possibility for similar linkages between oyster reefs and sandflat areas adjacent to the reefs. We have conducted a variety of field observations, field manipulations, and laboratory experiments to determine what predators may be utilizing oyster reefs and how their presence may affect infauna in adjacent habitats. Sampling over a variety of reefs emphasizes their importance as habitat for predatory fish and decapods. There is a trend towards lower abundance of certain infaunal groups near oyster reefs with evidence for stronger effects of predator exclusion immediately adjacent to a reef compared to several meters distant. Laboratory experiments confirm the potential for off-reef foraging by reef-associated predators. These results emphasize the need to consider habitats as interconnected units in management efforts.

NITROGEN EXCRETION BY THE PACIFIC OYSTER, *CRASSOSTREA GIGAS*; A CONTRIBUTOR TO ESTUARINE NUTRIENT CYCLING IN TOMALES BAY, CA. **Linda Righetti**, Romberg Tiburon Center, San Francisco State University, PO Box 855, Tiburon CA 94925.

Because of its importance as an aquaculture commodity, the filtering capacity and dietary requirements of the Pacific Oyster, *Crassostrea gigas*, have been studied in some detail. Most inquiries have focused on studies of food ingestion by the animal, and the portion of their intake that is converted to meat production. Very little attention has been given to the fate of excreted nutrients. Excreted matter may be returned to nutrient cycling systems, in such forms as ammonium (NH_4), which is readily taken up by phytoplankton and bacteria. This investigation found that *C. gigas* sampled from Tomales Bay (TB), California, provided with an excess of algal food excretes ammonium at a rate of 3.07 μg/g/h. Based on recorded biomass values for cultured oysters, *C. gigas* may be contributing ammonium to TB at a rate upward of 0.007 μM/h. The ambient phytoplankton population of 1 μg/l chlorophyll in TB is capable of using this NH_4, thus *C. gigas* excretion could account for up to 58% of N taken up by phytoplankton. Clearly the activity of filter feeders is important in determining the composition of available nutrients in the water column, impacting phytoplankton-based food webs in Tomales Bay. Future research will examine the extent to which nutrients may be removed from these systems, e.g. in the ingestion of diatoms resulting in the loss of unregenerated silica.

MUSSELS: SPACE MONOPOLISERS OR ECOSYSTEM-ENGINEERS? **Ray Seed**, School of Ocean Sciences, University of Wales, Menai Bridge, Anglesey, UK. LL59 5EY.

Mussels are extremely successful and widely distributed bivalve molluscs occurring in freshwater and estuarine habitats throughout the world and ranging from the high intertidal zone in coastal seas to mid ocean depths at sites of hydrothermal vent activity. This paper will explore some of the many reasons that underpin the evolutionary and ecological success of mussels and will briefly consider, by way of selected examples, the significance of their success to humans. As competitively dominant species mussels can potentially monopolise certain epibenthic communities with a consequent reduction in diversity of the primary space-occupying species. However, dense patches of mussels can dras-

tically modify the local environment, especially through biodeposition and the provision of additional habitat, features which serve to encourage species enrichment. The relative importance of mussels as space monopolisers and/or ecosystem engineers will provide a particular focus of this paper.

TWO STORIES OF PHYTOPLANKTON CONTROL BY BIVALVES IN SAN FRANCISCO BAY: THE IMPORTANCE OF SPATIAL AND TEMPORAL DISTRIBUTION OF BIVALVES. Janet K. Thompson, U.S. Geological Survey, Menlo Park, CA 94025.

The introduction of the Asian clam, *Potamocorbula amurensis*, into San Francisco Bay has resulted in changes to the food web within the northern bay (NB) but not within the southern bay (SB). *P. amurensis* invaded the bay in 1986, became the dominant member of the benthic community within one year in NB and within three years in SB. Large declines in phytoplankton biomass in NB appear to be due to "over-grazing" by *P. amurensis* populations which are estimated to filter the shallow reaches of NB in excess of twice a day. Because high turbidity restricts net positive primary production to the shallow reaches of NB and limits the net primary production in the deep areas of SB, shallow water grazing controls system-wide phytoplankton biomass throughout the system. SB phytoplankton biomass has not changed with the invasion of *P. amurensi*, despite similar density and biomass levels of *P. amurensis* in the deep water throughout the system. There are, however, large differences in the temporal and spatial distribution of shallow water *P. amurensis* in the NB and SB. Shallow water *P. amurensis* live 1 ½–2 years in the NB but only 8–9 months in the SB, and the annual phytoplankton bloom in SB occurs during the three month period when *P. amurensis* are absent from the shallow water.

GENETICS AND BREEDING

RESEARCH AND DEVELOPMENT ON SUMINOEGAKI, *CRASSOSTREA ARIAKENSIS*, FOR AQUACULTURE IN VIRGINIA, AND OTHER ACTIVITIES WITH NON-NATIVES. Standish K. Allen, Jr.,* Aquaculture Genetics and Breeding Technology Center, Virginia Institute of Marine Science, College of William & Mary, Gloucester Point, VA.

For several years, VIMS has been examining the biology and potential of non-native species for aquaculture development in the Chesapeake Bay. Earlier work has shown potential for *C. gigas* in higher salinity sites only and *C. ariakensis* generally throughout the Bay. With the goal of developing "put-and-take" aquaculture using sterile triploids, the Aquaculture Genetics and Breeding Technology Center has begun more extensive R&D on a number of fronts for *C. ariakensis*. Using stocks brought to the East Coast about 8 years ago, in 1999 we produced triploids for field trials, specifically to examine reversion and aspects of their marketability. From previous experiments, it is clear that reversion is a feature in triploid *C. ariakensis* as well. We have also attempted to produce tetraploid *C. ariakensis* with some difficulty. After dozens of attempts, some dozen or so putative tetraploids are in hand. We are also examining population genetic structure in collections throughout Southeast Asia. Early evidence points to discreet population structure among locales. Native Suminoegaki from southern and northern China were imported in 1999 and we produced F_1 diploids and triploids from the southern population. Larval culture for this species is still problematic in our hands and so we will be trying to optimize this fundamental step by working closely with collaborators in China. For *C. ariakensis* and also for more general work with non-natives, we have upgraded several key aspects of our physical plant, including development of a dedicated, land-based holding facility for long-term non-native research.

MICROSATELLITE MARKERS AS A TOOL TO STUDY REPRODUCTIVE SUCCESS IN THE PACIFIC OYSTER, *CRASSOSTREA GIGAS* (THUNBERG), CROSSED UNDER CONTROLLED HATCHERY CONDITION. Pierre Boudry and **Bertrand Collet,** Laboratoire IFREMER de Génétique et Pathologie, BP133, 17390 La Tremblade, France; **Florence Cornette, Véronique Hervouet** and **François Bonhomme,** Laboratoire Génome, Populations, Interactions, 1 quai de la Daurade, 34200 Sète, France.

Oysters, like many marine species have a very high fecundity. Previous studies have shown that populations, from both hatcheries and the natural environment, have very low *Ne/N* ratios. These observations reveal high variation in reproductive success. In order to study individual reproductive success under controlled conditions, we used microsatellite markers to quantify parental contributions in *in vitro* crosses (5 males and 5 females) of *Crassostrea gigas*, the Pacific oyster. High polymorphism of the microsatellites (more that 50 alleles per locus) eased the parentage identifications. The results of a cross allowing gametic competition were compared with the results from a second cross where the gametes of the same parents were kept separate for each parental combination until after fertilization. The progeny were then sampled at different stages of development and the parental contributions determined to follow their evolution through time. Despite the fact that equal numbers of gametes were mixed for each male and each female, the contributions of these parents to the resulting progeny was highly unbalanced at both larval and juvenile stages in both crosses. We demonstrated that variation in individual reproductive success is due to both spermatic competition and selective phenomena at early stages.

BEAD-BASED GENOMICS TECHNOLOGIES AT LYNX: APPLICATIONS FOR PACIFIC OYSTER BREEDING. **Ben Bowen,** Lynx Therapeutics, Inc., Hayward, CA 94545.

Lynx has developed a method (Megaclone™) for cloning amplified DNA fragments on the surface of 5-micron plastic beads rather than in *E. coli*. Beads harboring differentially expressed genes can be identified rapidly using a fluorescence activated cell sorter in a process called Megasort™. An automated procedure for determining 16–20 bases of signature sequence from upto one million beads simultaneously (Massively Parallel Signature Sequencing or MPSS™) has also been devised. These signature sequences assign an identity to each bead-based clone, and, in many cases, allow matching of each clone in a bead array with genes in a sequence database. Finally, a new application of Lynx's bead-sorting technology (Megatype™) is being developed to identify polymorphic genomic DNA fragments that correspond to alleles present at different frequencies in two phenotypically distinct populations. I will discuss applications of these technologies for the genetics and breeding of Pacific oysters, especially understanding the phenomenon of heterosis or hybrid vigor.

TRANSFECTION OF EASTERN OYSTER EMBRYOS. **John T. Buchanan,*** Department of Oceanography and Coastal Sciences, Louisiana State University, Baton Rouge, LA 70803; **Amy D. Nickens** and **Terrence R. Tiersch,** Aquaculture Research Station, Louisiana State University Agricultural Center, Baton Rouge, LA 70820; **Richard K. Cooper,** Department of Veterinary Science, Louisiana State University, Baton Rouge, LA 70803.

There is a need for research in disease resistance and microbial elimination in eastern oysters, *Crassostrea virginica*. Gene transfer research may lead to advances in this area, and a means of selecting transfected larvae would be useful. We transfected 3-h-old embryos with the bacterial gene aminoglycoside phosphotransferase II (*neo*^{r}), which confers resistance to neomycin and related antibiotics such as G418. The antibiotic G418 was examined as a potential selective agent. A neutral red assay was used to determine survival after 48-h exposure to various concentrations of G418 (0 to 4 mg/ml). We examined the effects of electroporation and chemically mediated transfection (SuperFect™; Qiagen) of 3-h-old embryos on survival to D-stage larvae. DNA alone was found to have no effect on survival ($P > 0.05$). For electroporation, we found that increasing voltage and pulse duration decreased survival ($P < 0.05$). Chemically mediated transfection did not significantly affect survival ($P = 0.5172$). Transgenic larvae were produced by electroporation or chemically mediated transfection of 3-h-old oyster embryos with *neo*^{r}. These embryos were reared for 24 h and exposed to G418 at 0.3 mg/ml for 48 h. Significant differences in survival between transfected and nontransfected larvae were detected for electroporation ($P = 0.0147$) and chemically mediated transfection ($P = 0.037$). This study documents the successful insertion and expression of foreign DNA in eastern oyster larvae.

ESTIMATION OF NARROW-SENSE HERITABILITY FOR LARVAL AND JUVENILE GROWTH TRAITS IN SELECTED AND UNSELECTED SUB-LINES OF EASTERN OYSTERS, *CRASSOSTREA VIRGINICA*. **Christopher V. Davis,*** Darling Marine Center, University of Maine, 193 Clarks Cove Road, Walpole, ME 04573, USA.

When embarking on a selective breeding program, prior knowledge of the heritability for the selected trait is useful in developing an optimal breeding design. Narrow-sense estimates of heritability describe the proportion of phenotypic variation due to additive gene effects. These estimates are useful in predicting potential gains that may result from exploitation of additive genetic variance in selection programs. The goal of this study was to determine the narrow-sense heritability for juvenile growth traits in hatchery propagated lines of eastern oysters, *Crassostrea virginica* (Gmelin 1791) selected for rapid growth.

Heritabilities for growth traits were determined using half-sib analysis of twenty-five families produced from 5 × 5 diallele crosses. Estimates were made from a population having undergone three generations of selection for rapid growth and from an unselected control sub-line. Both sub-lines were originally derived from a common source population. Sire-based estimates of heritability for larval shell length were 0.44 (±0.14) and 0.14 (±0.07) in the selected and unselected control sub-lines, respectively. Corresponding estimates for live weight at 7 months were 0.51 (±0.15) and 0.10 (±0.05), respectively. These results suggest that selective breeding efforts may significantly increase growth rates in oysters, although the magnitude of improvement may vary considerably, depending on the degree of exploitable additive genetic variance within the population.

ANALYSIS OF GENETIC DIVERSITY IN A COMMERCIALLY IMPORTANT LINE OF OYSTERS SELECTED FOR FAST GROWTH. **Daniel DenDanto,*** University of Maine, Orono, ME 04469; **Bonnie L. Brown,** Virginia Commonwealth University, Richmond, VA 23284; **Chris Davis,** Pemequid Oyster, Waldaboro, ME 04572; **Irving Kornfield,** University of Maine, Orono, ME 04469.

Levels of heterozygosity and allelic diversity at discrete microsatellite loci are compared among two hatchery derived lines and a wild population of Eastern Oyster, (*Crassostrea virginica*) to assess the effects of "selection" for fast growth among the hatchery lines. Wild samples and "selected" experimental cohorts of "Milford" and "Flowers" oyster lines are evaluated for generational loss of genetic variation within and between the groups. A commercially important line of oyster, improved for growth on the Maine coast, is investigated for changes in its genetic background

over four generations of selection and over larvel and juvenile phases of an F_4 cohort. Comparison of these hatchery lines to wild populations of oysters in the Gulf of Maine allows for a much needed evaluation of genetic consequences incurred during the selection experiments for fast growth. Information on the genetic change across life history stages within the "Flowers" F_4 cohort addresses unique deviations from population genetic expectations as observed by other investigators for this genus.

PATTERNS OF NUCLEOTIDE VARIATION AT THE *GPI* LOCUS IN THE BLUE MUSSEL *MYTILUS EDULIS*. **Matthew P. Gordon*** and **Paul D. Rawson**, School of Marine Sciences, Murray Hall, University of Maine, Orono, ME 04469.

Glucose-6-phosphate isomerase (GPI) is an enzyme that functions as a branch point between the glycolytic pathway and the pentose shunt pathway. In many coastal marine taxa along the Atlantic Coast of North America there is a high degree of allelic polymorphism as well as concordant patterns of allelic distribution for this enzyme. These concordant patterns suggest that adaptation is important in the maintenance of variation at the *Gpi* locus. For *Mytilus edulis*, Hall (1985) demonstrated temperature related kinetic differences between two common *Gpi* alleles, $GPI^{1.00}$, which is most common in the Mid-Atlantic, and $GPI^{0.96}$, which increases in frequency with latitude. This evidence is consistent but not conclusive with regard to the hypothesis that *Gpi* is thermally adapted in *M. edulis*. The objective of this study was to examine DNA sequence variation for *Gpi* in *M. edulis* to further investigate the role of selection in the maintenance of variation at this locus. From preliminary sequence information obtained by using RT-PCR and 5′/3′ Race methodologies we have designed primers that amplify complete coding segments of *M. edulis Gpi*. Individual mussels from Merrick, NY and Walpole, ME have been allotyped at GPI by electrophoresis. From individuals homozygous for either $GPI^{1.00}$ or $GPI^{0.96}$ we have isolated complete coding sequences. We will present an analysis of patterns of nucleic and amino acid variation among the sequences using a *Gpi* sequence from the congener *M. trossulus* as an outgroup.

ANEUPLOIDY IN THE PACIFIC OYSTER, *CRASSOSTREA GIGAS* THUNBERG AND ITS EFFECTS ON GROWTH. **Ximing Guo,[1]* Guofan Zhang,[2] Brenda J. Landau,[1] Louise English,[1]** and **Yongping Wang,[3]** [1]Haskin Shellfish Research Laboratory, Rutgers University, 6959 Miller Avenue, Port Norris, NJ 08349, USA; [2]Key Laboratory of Aquacultural Ecology, Dalian Fisheries University, Dalian, Liaoning 116025, PRC; [3]Experimental Marine Biology Laboratory, Institute of Oceanology CAS, Qingdao, Shandong 266071, PRC.

We previously described the incidental induction of aneuploids in the Pacific oyster, *Crassostrea gigas* Thunberg, from tetraploid and triploid production. Here we report the intentional production of aneuploids and isolation of trisomic families, with observations on their growth performance. The first generation of aneuploids was produced from triploid × diploid crosses. Two types of crosses were made: diploid ♀ × triploid ♂ (DTA) and the reciprocal triploid ♀ × diploid ♂ (TDA) crosses. DTA crosses were highly effective in producing aneuploids, and 80–95% of the DTA progeny were aneuploids as determined by chromosome counts at one year of age. Aneuploid conditions included 2n + 1, 2n + 2, 2n + 3, 3n − 2, and 3n − 1. TDA crosses produced fewer aneuploids (16–20%) and more triploids (20–53%) than DTA crosses. Aneuploids as a group are significantly smaller in size than normal diploids. DTA progeny with an approximate diploid DNA content were separated using flow cytometry and considered putative trisomics. The putative trisomics were crossed with normal diploids in single-pair matings. Sixty putative trisomic families were produced, and 20 of them were confirmed as trisomic families using chromosome counts of embryos at the 2-cell stage. In most families, the frequency of trisomics sharply declined, from about 50% at the 2-cell stage to 5–25% at one year of age, possibly due to mortality or chromosome loss. In some families, the trisomics remained at 40–61%. Trisomic oysters are smaller on average than normal diploids in most families, but not different from normal diploids in others. Results of this study show that the Pacific oyster can tolerate aneuploidy up to 15% of its genome. Aneuploids as a group have growth retardation, but certain aneuploid conditions grow as well as normal diploids.

IMPROVING PACIFIC OYSTER BROODSTOCK THROUGH CROSSBREEDING. **Dennis Hedgecock,*** University of California, Davis, Bodega Marine Laboratory, Bodega Bay, CA 94923-0247; **Jonathan P. Davis,** Taylor Resources, Inc., 701 Broad Spit Rd., Quilcene, WA 98376.

Controlled crosses among inbred lines of the Pacific oyster *Crassostrea gigas* reveal much hybrid vigor or heterosis for larval and adult growth rate. Evidence for a large genetic load in this oyster suggest that hybrids are superior because they inherit dominant alleles that mask deleterious recessive mutations in many functional genes. Physiologically, hybrids appear to have greater efficiencies than inbreds in energy and protein metabolism and feeding. How growth or yield of hybrid oysters compares to that of farmed Pacific oysters has been the focus of a project funded by the USDA's Western Regional Aquaculture Center since 1993. Initial large-scale comparisons of inbred and hybrid with farmed oysters suggest that crossbreeding can improve commercial broodstocks. Inbred offspring, which were made by mating siblings from the first selected generation of oysters in the Molluscan Broodstock Program, Hatfield Marine Science Center, Newport, OR, show inbreeding depression, as expected. Growth trials comparing WRAC hybrids and MBP select families are in progress. Hybrid larvae produced in 1998 at the Taylor Shellfish Hatchery, Quilcene, WA, grew faster and set 4–5 days earlier than larvae

from commercial control spawns. Body-size data for these hybrids, which are growing on long-lines in Samish Bay, WA, will be obtained in December 1999. Despite the promise indicated by evidence for heterosis and WRAC results to date, commercial implementation of crossbreeding will require testing crosses among hundreds if not thousands of inbred lines. As it is not possible to rear such a large number of groups in commercial culture facilities, very early physiological or molecular indicators of hybrid performance are needed to improve the efficiency of testing. Differences in respiration between inbred and hybrid oysters, for example, are evident at the early trochophore stage; comparisons of respiration and protein turnover among different hybrids at the trochophore stage are therefore planned. Patterns of early gene expression are also being explored for their potential use in identifying elite inbred lines for hybrid oyster production.

MICROSATELLITE ANALYSIS OF TRISOMIC FAMILIES IN THE PACIFIC OYSTER, *CRASSOSTREA GIGAS* THUNBERG. **Sophie Hubert,[1]* Louise J. English,[2] Brenda J. Landau,[2] Ximing Guo,[2]** and **Dennis Hedgecock,[1]** [1]Bodega Marine Laboratory, University of California at Davis, P.O. Box 247, Bodega Bay, CA 94923; and [2]Haskin Shellfish Research Laboratory, Institute of Marine and Coastal Science, Rutgers University, 6959 Miller Avenue, Port Norris, NJ 08349.

Trisomy (2n + 1) is an aneuploid condition where one chromosome is represented by three copies instead of the normal two copies. Change in copy number may affect the expression of genes located on the trisomic chromosome and therefore, analysis of trisomics may be useful for the chromosomal assignment of markers and quantitative trait loci. We produced trisomic families in the Pacific oyster, *Crassostrea gigas* Thunberg, and tested microsatellite markers for trisomic identification and analysis. Trisomic families were produced in two steps. First, diploid × triploid crosses were made, producing a mixture of normal diploids, triploids, trisomics and other aneuploids. Individuals with an approximate diploid DNA content were separated with flow cytometry and considered as putative trisomics. Then putative trisomics were crossed with each other or with normal diploids in single-pair matings. Sixty putative trisomic families were produced, and 20 of them were confirmed as trisomic families using chromosome counts of embryos at 2-cell stage. Parents from 16 trisomic families were screened with 14 microsatellite markers. Tri-allelism (3 alleles/locus/individual) was observed at three loci in six trisomic families. The tri-allelism was found only in the putative trisomic parent, not in normal diploids. One locus was tri-allelic in three of the 16 families, suggesting that the chromosome carrying this locus may be over-represented among the trisomic families. Progeny from the trisomic families are being analyzed for confirmation of trisomic inheritance. Results so far indicate that trisomic families can be readily produced, and microsatellite markers are useful in trisomic identification because of their high polymorphism.

NUCLEAR CONTROL OF SEX RATIO BIAS IN THE MUSSEL *MYTILUS EDULIS*. **Ellen Kenchington*** and **Liqin Cao,** Department of Biology, Dalhousie University, Halifax, Nova Scotia B3H 4J1, Canada; **Eleftherios Zouros,** Institute of Marine Biology of Crete, Greece.

Previous studies have shown that in pair-matings of *Mytilus edulis*, *M. trossulus* and *M. galloprovincialis* there can be a large sex-ratio bias in favor of either males or females. The degree of bias is a characteristic property of the female parent, as matings of the same female with different males produce the same sex ratio, but matings of the same male with different females produce different sex ratios. All three species possess the unusual feature of doubly uniparental inheritance of mitochondrial DNA (mtDNA), *i.e.*, they contain two distinct types of mtDNA, one that is transmitted patrilinearly (the M type), and one that is transmitted matrilinearly (the F type). This coupling of sex and mtDNA inheritance raises the possibility that a female's sex-ratio is under the control of the female's mtDNA. Here we present data from controlled pair matings that are incompatible with this hypothesis, but are consistent with a nuclear control of sex ratio.

GROWTH, SHELL MORPHOLOGY, REPRODUCTIVE PHYSIOLOGY, AND MOLECULAR GENETIC ANALYSIS OF TASMANIAN PACIFIC OYSTERS, *CRASSOSTREA GIGAS*, IN WASHINGTON STATE. **Manfred Kittel*** and **Kenneth K. Chew,** School of Fisheries, University of Washington, Seattle, WA 98195.

Controlled introductions of shellfish populations may be economically beneficial to the aquaculture industry by providing desirable traits and improved productivity through hybrid vigor. A small number of deep-cupped Pacific oysters (*Crassostrea gigas*) was transferred from a Tasmanian shellfish hatchery to the state of Washington in 1994. Oysters were spawned artificially in two separate mass spawns with maximum effective population sizes (N_e) of 18 and 6.4, respectively. The resulting F_1 oysters were compared to control *C. gigas* of local origin with respect to overall survival, growth rate, shell morphology, gonadal maturation, and glycogen storage. A molecular genetic analysis was performed to determine the species status of the introduced oysters, investigate the possible loss of genetic variability due to the founder effect, and to develop a DNA-based molecular population marker.

Results from this study indicate that the Tasmanian F_1 oysters experienced a 40% reduction in cumulative mortalities and attained significantly greater length, weight, and volume than controls. There was no decrease in heterozygosity or polymorphism but allelic variation was reduced by 28% due to the loss of several

rare alleles. Restriction of a 2,100 bp mitochondrial (mt) ribosomal DNA segment (including ITS-1 and ITS-2) with 45 restriction endonucleases did not produce population-specific haplotypes. However, 50% of a small sample (N = 14) of Tasmanian F_1 oysters were characterized by a C → T transition at one specific nucleotide position within a 524 bp PCR-amplified DNA fragment of the mt cytochrome *b* locus.

THE MOLLUSCAN BROODSTOCK PROGRAM—IMPROVING PACIFIC OYSTER BROODSTOCK THROUGH GENETIC SELECTION. Chris J. Langdon,* Dave P. Jacobson, and **Ford Evans,** Hatfield Marine Science Center, Oregon State University, Newport, OR 97365; **Mike S. Blouin,** Zoology Department, Oregon State University, Corvallis, OR 97331.

The Molluscan Broodstock Program (MBP) was established in 1995 to improve yields of Pacific oysters on the West Coast, U.S., by genetic selection. Currently, about 400 full-sib families have been produced and planted at commercial sites in West Coast states.

The performance of top-performing families (expressed in terms of live weight per bag) is up to five times greater than that of poorer performing families. There is a strong environment-genetic interaction effect on relative family performance, although "generalist" families are evident that perform well across a range of culture environments.

Yields (live weight per bag) of progeny from crosses among top-performing founder MBP families are significantly greater (Fisher's PLSD test; $p < 0.05$) than those of progeny from non-selected "wild" oysters but not significantly different from those of industry stocks. The heritability value for yield was estimated to be 0.54, indicating genetic selection should result in significant, long-term improvements in commercial oyster production.

EVIDENCE OF ABSORPTION EFFICIENCY DIFFERENCES IN TWO SUBPOPULATIONS OF *CRASSOSTREA GIGAS*. A FIRST APPROACH OF THEIR AMYLASE GENE POLYMORPHISM. J. Moal, J. F. Samain,* and **J. Y. Daniel,** Laboratoire de Physiologie des Invertébrés, Ifremer Centre de Brest BP 70, 29280 Plouzané, France; **P. Boudry,** Laboratoire de génétique Ifremer Centre de La Tremblade; **S. Bougrier,** CREMA Ifremer-CNRS L'Houmeau; **D. Sellos** and **A. Van Wormhoudt,** MNHN, laboratoire de biologie marine, BP 225, 29182, Concarneau.

A G1 population from three 5 × 5 crosses of *Crassostrea gigas* oysters from three origins was reared at low density and was fed in the same controlled conditions for two years to study origin of growth variability in the frame of the European genetic programme GENEPHYS (Genetic and Physiology). Relationships between digestive enzyme activities, ingestion, absorption efficiency and polymorphism of amylase genes, were studied on individuals of this *C. gigas* generation. Two sub populations among the 60 individuals were evidenced, based on two different relationships observed between absorption efficiency, ingested food, and digestive amylase activities. A first approach showed differences in the relationships between ingestion, enzyme activities, and in Michaelis constants (Km). A study on amylase genes, as a model, was undertaken to study a possible genetic origin of these physiological and catalytic traits. Gene structure was determined using different sets of primers deduced from the amylase cDNA sequence, previously determined. Two different amylase genes (A and B) were characterized through their differences in nucleotide sequences. A first approach of individual polymorphism of amylase genes was performed on the two physiologically characterized subpopulations, using PCR and RFLP on the genomic DNA of individuals, and specific primers of both genes. Two different variants were observed for gene A and three for gene B corresponding to one or two EcoR1 restriction sites. Differences in frequencies of the different observed variants were evidenced between the two oyster subpopulations. These first results and interest of such an approach to identify functional genetic markers for selection are discussed.

GENOTYPE DEPENDENT DIFFERENCES IN FEEDING RATES AND GROWTH IN OYSTER LARVAE. Douglas A. Pace and **Donal T. Manahan,** Department of Biological Sciences, University of Southern California, Los Angeles, CA 90089.

A major biological question concerning heterosis is the physiological explanation(s) for the observed differences in growth. Differences in growth rates and feeding rates on algae were measured for larvae of the Pacific oyster, *Crassostrea gigas*, that had relatively high (hybrids) and low (inbreds) levels of heterozygosity. In 4 independent experiments, involving the crossing of 2 or more inbred lines, heterosis for growth was observed. Levels of heterozygosity also had a significant effect on size-specific feeding rates of larvae. The slopes of the relationships between the increase in algal clearance rates with larval growth (shell length) were the same between hybrid and inbred larvae (ANOVA, $p > 0.05$). However, there was a significant difference in the y-intercept values between the two groups (ANOVA, $p < 0.001$), such that at any given shell length hybrid larvae were feeding faster than their inbred counterparts. For instance, at a shell length of 280 μm, hybrid larvae had a mean clearance rate (based on 4 different experiments) that was 95% higher than inbred larvae. Additional

measurements showed that rates of oxygen consumption and citrate synthase (index of mitochondrial activity) were the same for both inbred and hybrid larvae. We conclude that faster growing hybrid larvae have higher size-specific feeding rates, but similar metabolic rates, and so have a higher scope for growth than inbred larvae.

DEVELOPMENT OF MOLECULAR MARKERS FOR CONSTRUCTING A GENETIC LINKAGE MAP OF THE EASTERN OYSTER *CRASSOSTREA VIRGINICA*. **Kimberly S. Reece*** and **Wenda L. Ribeiro,** Virginia Institute of Marine Science, The College of William and Mary, Gloucester Point, VA 23062; **Patrick M. Gaffney,** College of Marine Studies, University of Delaware, Lewes, DE 19958; **James Pierce,** University of the Sciences in Philadelphia, Philadelphia, PA 19104.

The oyster diseases Dermo and MSX continue to plague the eastern oyster *Crassostrea virginica*, dramatically reducing wild harvests and discouraging the establishment of aquaculture operations in affected waters. A potential solution to this problem is development of genetically improved disease-resistant strains of *C. virginica* that can grow to market size despite disease challenge. One means of accelerating the selective breeding process is to identify genetic markers associated with traits such as disease resistance or growth rate. The goal of this project is to develop genetic markers for constructing a linkage map and to eventually identify markers associated with specific traits. To date six allozyme and nine nuclear DNA polymorphisms have been scored in parents and F1 progeny of a panel of ten reference families of known pedigree. We are currently developing additional DNA markers (single-copy, micro- and minisatellite). 768 *C. virginica* clones from a small insert genomic DNA library have been partially sequenced, providing ≈700,000 bp for marker development. Both unknown sequences and putative coding regions are being screened for polymorphisms by denaturing gradient gel electrophoresis analysis of PCR amplified fragments. PCR primers have also been designed to anneal to regions flanking identified repeat sequences. Amplification reactions have been optimized for analysis of size variation on the automated DNA sequencer at fourteen repetitive sequence loci, which includes one tetra-, three tri- and four di-nucleotide microsatellite sequences. In addition, we are developing a 384-well microtiter plate PCR-based assay to facilitate identification of specific short tandem repeats (STRs). Preliminary screening of the genomic library has identified a number of trinucleotide STR candidates. Genotypes for the allozyme and nuclear DNA loci are being generated for the parents and 35–40 F1 individuals of the ten reference families in order to construct a preliminary genetic linkage map.

GENETIC CHARACTERISTICS OF WILD AND CULTURED MUSSELS, *MYTILUS EDULIS* AND *MYTILUS TROSSULUS* IN PRINCE EDWARD ISLAND (GULF OF ST. LAWRENCE). **Réjean Tremblay,*** Centre Aquicole Marin, MAPAQ—Université du Québec à Rimouski, Grande-Rivière, Qué., G0C 1V0; **Thomas Landry,** Gulf Fisheries Center, DFO, Moncton, N.B., E1C 9B6; **Bruno Myrand,** Station Technologique Maricole des Îles-de-la-Madeleine, MAPAQ, Cap-aux-Meules, Qué., G0B 1B0; **Jean-Marie Sévigny,** Institut Maurice-Lamontagne, MPO, Mont-Joli, Qué., G5H 3Z4.

For the first time, *Mytilus trossulus* was observed in populations generally considered as totally *Mytilus edulis* in Prince Edward Island but at low level (only 9.7% in 1997 and 2% in 1999). Furthermore, we observed in both years (1997 and 1999), genetic differentiations in *Mytilus edulis* populations from different sites in Prince Edward Island, particularly between wild and cultivated mussels. The genetic differences, observed by electrophoretic data on multiple loci, were not related to allelic frequencies, but to the genotype structure, particularly to the proportion of heterozygous individuals. We observed that off-bottom cultivated mussels exhibited an important heterozygote deficiency, comparatively to wild mussels, in sites where no cultures were practised. As gene flow is most likely important between sites, we suggest that the genetic differentiation was in relation with culture practice. These results would be discussed in relation with our studies in Magdalen Island, where we have demonstrated the energetic advantages of more heterozygous individuals and the impact of suspension-cultured methods on mean heterozygosity of mussel populations. Finally, gene flow of *Mytilus edulis* is discussed between the 250 km separating the Prince Edward Island and the Magdalen Island.

MICROSATELLITE VARIATION IN GEODUCK CLAMS (*PANOPEA ABRUPTA*) IN PUGET SOUND, WASHINGTON. **Brent A. Vadopalas,* Are Strom,** and **Paul Bentzen,** School of Fisheries, University of Washington, Seattle, WA 98105.

Population differentiation is often assumed to be non-existent among marine invertebrate species with high dispersal potential due to a protracted pelagic larval phase. Using high resolution DNA microsatellites and other molecular markers, however, genetic differences between populations of some marine invertebrates have been demonstrated on both macro- and microgeographic scales. Similar analyses of geoduck clam population genetics are problematic, in that these extremely long-lived clams occur in contagious distributions in Puget Sound, Washington, with each patch comprised of many overlapping generations. The effects of temporal variation and sweepstakes recruitment must be considered in the interpretation of spatial genetic variation in pre-

sumed neutral microsatellite loci. Both age and genetic data are necessary to examine this hypothesis.

Microsatellite allele frequency data were collected from 100 individuals each from semi-isolated populations in Hood Canal, South Puget Sound, and the Straight of Juan de Fuca, in addition to an outgroup from Southeast Alaska. The populations were screened for allelic variation using seven tetranucleotide and three dinucleotide microsatellite loci developed via magnetic bead hybridization selection methods. Puget Sound samples were aged by counting hinge plate annuli on thin-sections from the right valve. A significant deficiency of heterozygotes was detected at many loci necessitating the use of alternate, less powerful tests of genetic differentiation independent of assumptions of Hardy-Weinburg equilibrium. Analysis of age and microsatellite data may provide valuable insight into the genetic population structure of this species.

CHROMOSOMAL LOCATION OF SOME REPETITIVE DNA IN *CRASSOSTREA* OYSTERS AS DETERMINED BY FISH. **Yongping Wang,[1]* Zhe Xu,[2] Ximing Guo,[2] James C. Pierce,[3]** and **Patrick M. Gaffney,[4]** [1]Experimental Marine Biology Laboratory, Institute of Oceanology CAS, Qingdao, Shandong 266071, PRC; [2]Haskin Shellfish Research Laboratory, Rutgers University, 6959 Miller Avenue, Port Norris, NJ 08349; [3]Department of Biological Sciences, University of Science in Philadelphia, Philadelphia, PA 19104; [4]College of Marine Studies, University of Delaware, Lewes, DE 19958.

Characterization and identification of chromosomes are needed for several types of genomic analyses and mapping. Although oysters have a low haploid number of 10, oyster chromosomes are difficult to characterize because of their similarities in size and shape. Traditional banding techniques in oysters have been difficult and unreliable. Fluorescence in situ hybridization (FISH), on the other hand, may provide a powerful tool for the identification and physical mapping of oyster chromosomes. We tested FISH on oyster chromosomes with several repetitive DNA sequences using chromosomes from early embryos. All probes were made by PCR amplification and incorporation of DIG-11-dUTP. Metaphase chromosomes prepared from early embryos were adequate for use in FISH analysis. In *C. virginica*, an anonymous repetitive DNA fragment produced strong signals on several chromosomes, although some locations were not as stable as others. Two short repetitive sequences (156 and 283 bp) hybridized to all regions of all chromosomes, suggesting that these two elements are dispersed throughout the genome. In *C. gigas*, a short repetitive sequence was mapped to centromeric regions of 5–7 chromosomes. FISH signals were small or weak for all repetitive sequences studied so far, possibly suggesting that *Crassostrea* oyster genomes have relatively little repetitive DNA. Nevertheless, this study shows that FISH with repetitive DNA is useful for chromosome identification.

INVERTEBRATE FISHERIES

THE BRITISH COLUMBIA FISHERY FOR NORTHERN ABALONE, *HALIOTIS KAMTSCHATKANA*: MANAGEMENT FROM INCEPTION TO CLOSURE AND BEYOND. **Bruce E. Adkins,** Fisheries and Oceans Canada, Pacific Biological Station, Nanaimo, B.C. V9R 5K6 Canada.

Northern abalone (*Haliotis kamtschatkana*) have been harvested by First Nations in British Columbia for food, social and ceremonial purposes since pre-recorded time. While recreational and commercial abalone fisheries have occurred in British Columbia since as early as 1900, these were small and largely unregulated up to 1972 when a directed commercial dive fishery began.

The commercial abalone fishery, which developed during the 1970's, typifies an emerging fishery. Annual landings increased rapidly to 433 tonnes in 1978 but then declined quickly to less than 100 tonnes by 1981 and then to 47 tonnes in 1985 as increasingly restrictive management measures were applied in an attempt to establish sustainable harvests. Despite the restrictive management measures, however, abalone stocks measured at key index sites continued to decline during the course of this fishery. As a result, in 1990, conservation concerns led Fisheries and Ocean Canada to close the abalone fishery to harvesting by all user groups.

Aboriginal, commercial and recreational fisheries have remained closed as assessment surveys since 1990 have shown a further decline in abalone abundance at key index sites. While this is likely biologically related, illegal fishing is considered to be a contributing factor.

Concerns with respect to the continued declines in abalone stocks since the fishery closure has resulted in the northern abalone being designated as "threatened" in British Columbia by the Committee on Status of Endangered Wildlife in Canada (COSEWIC).

A stock rehabilitation initiative currently being developed for northern abalone in British Columbia is discussed.

The abalone fishery, the management measures and the post closure activities are described.

STOCK ASSESSMENT AND MANAGEMENT OF RED SEA URCHINS (*STRONGYLOCENTROTUS FRANCISCANUS*) IN WASHINGTON. **Alex Bradbury,*** Washington Department of Fish and Wildlife, Point Whitney Shellfish Laboratory, Brinnon WA 98320.

Washington's commercial red sea urchin fishery began in 1971. The first regulations in 1977 included a restricted winter season, area rotation, minimum and maximum size limits, and mandatory logbooks. Exploratory surveys began in 1978, and annual surveys at index stations on the commercial beds began in 1984. Annual landings peaked at 3,658 t in 1988, followed by restricted seasons

and limited entry which reduced fleet size by 67%. Until 1993, managers made *ad hoc* adjustments to season length based on the observed trends in urchin density and size at index stations. Beginning in 1993, a size-structured model based on survey data was used to recommend harvest rates in five management regions. Biomass has been estimated using one of four methods: 1) Underwater video scans at systematically-spaced sites; 2) Adaptive Cluster Sampling (ACS) dive surveys, modified to permit a fixed sample size; 3) Change-In-Ratio (CIR) estimates based on known catch and the observed change in urchin density from surveys; and 4) Leslie estimates based on declining CPUE. The CIR and Leslie methods are problematic because only high harvest rates provide suitable data for making biomass estimates. Similarly, long-term stability in CPUE has prevented the use of surplus production models. Direct sampling methods, such as video or ACS, are currently considered the only reliable ways to estimate biomass. Funding problems ended surveys in most regions in 1995, and the last direct biomass estimate occurred in 1997. In 1998, TACs were reduced 15% from the 1997 levels as an arbitrary precaution in the absence of survey data.

SHRIMP FISHERIES AND MANAGEMENT IN HOOD CANAL AND PUGET SOUND. Therese A. Cain* and **Jay G. Odell,** Washington Department of Fish and Wildlife, Point Whitney Shellfish Laboratory, Point Whitney Road, Brinnon, WA 98320.

Shrimp stocks in the Hood Canal and Puget Sound have supported important shrimp fisheries for much of the past century. This paper describes historical trends in relative stock abundance of spot shrimp in Hood Canal as well as the present status of those stocks. These trends are illustrated using data from preseason test fisheries conducted to provide annual estimates of total allowable catch. A 1994 federal court order requires co-management to provide for equitable state/tribal sharing of Hood Canal shrimp between the state recreational fishery and tribal commercial fishery. Methods the Washington Department of Fish and Wildlife uses to provide recreational harvest opportunity while meeting allocation and conservation requirements are described.

Puget Sound (excluding Hood Canal) shrimp fisheries are managed with a more passive management scheme. This presentation will discuss trends in landings and the current status of the state commercial and recreational fisheries, and the tribal commercial fishery which has been developing since 1995. Because of rapidly increasing participation in the state commercial fishery, the Emerging Fisheries Act was implemented in 1994 to reduce the state commercial shrimp pot fleet from 73 to 18 boats and the shrimp trawl fleet from 15 to 10 boats. In 1996, WDFW began comanaging the shrimp resource with treaty tribes, and quotas were established for pandalid shrimp in areas of Puget Sound where fishing previously occurred. Quotas were based on historical harvests with adjustments based on recent fishery performance. A description of other methods used to manage these fisheries is also presented.

APPLICATION OF HYPOTHESIS TESTING AND POWER ANALYSIS IN THE PUGET SOUND CRAB FISHERY: CLOSURE DECISIONS WITH CONFIDENCE. Jennifer Cahalan,* Washington Department of Fish and Wildlife, Point Whitney Shellfish Laboratory, Brinnon, WA 98320.

Traditional fisheries' methods often rely on point estimators to determine fishery decisions. This includes closure of fishing areas due to the presence of an undesirable characteristic, such as a toxin accumulation, fish condition, presence of disease, or low indexes of population abundance. In many cases, a hypothesis test, which considers the probabilities of making correct and incorrect decisions, is better suited to the problem than the use of a single point estimate of the characteristic in question. The Dungeness crab fishery in Puget Sound is managed through a combination of size limits, daily recreational harvest limits, and area closures during periods when the crabs are in a soft-shell condition. In order to determine the appropriate soft-shell closure periods sampling is conducted in areas of fishing activity. When the point estimate of the proportion of crabs in soft-shell condition exceeds a critical point, the fishery is closed. A sample design for shell condition testing was developed to test the null hypothesis that the sampled crabs came from a population of soft-crabs. Sample size was determined beforehand to control both the probability of opening the fishery when the crabs are soft (type I error), and the probability of closing the fishery when the crabs are hard (type II error). The hypothesis test allows us to control sample size so that the probability of making a wrong decision is within an acceptable range. Point estimates do not provide this additional information and probability of wrong decisions cannot be readily assessed. Given that sample data has uncertainty, this hypothesis test can improve fishery management decisions.

MANAGEMENT OF INTERTIDAL BIVALVES IN PUGET SOUND, WASHINGTON. James I. Child,* Squaxin Island Tribe, Natural Resources Department, 2752 Old Olympic Highway, Shelton, WA 98584; **William W. Campbell,** Washington State Department of Fish and Wildlife, Point Whitney Shellfish Laboratory, 1000 Point Whitney Road, Brinnon, WA 98320.

With over 35,000 acres of public beach in Washington State, intertidal clams and oysters provide a high source of economic and social benefit for recreational and commercial harvesters. Western

Washington Treaty Tribes reserved the right to harvest shellfish under the Stevens Treaties in the 1850's. A 1994 federal district court ruling recognized the Tribes' right to harvest up to 50% of the harvestable biomass of shellfish found within their usual and accustomed fishing grounds. Intertidal clams and oysters are co-operatively managed in the State of Washington between the affected Treaty Tribes in a given region and the State of Washington. There are a total of 8 intertidal management regions encompassed within the waters of Puget Sound.

With Treaty rights to shellfish established and an increasing public demand for shellfish, there is a need for a more intensive beach management strategy. This paper will give a brief overview of the management activities directed at beach-by-beach management and identify some alterations from past State wide management practices. Processes include a State wide bivalve agreement, regional annual management plans, population estimation, calculation of "harvestable biomass", fishery monitoring and catch reporting.

Using a regional approach to intertidal management has allowed for identification of the differing biological requirements that exist from region to region. In various regions, the need to utilize adaptive management practices to meet the biological requirements of the species is being considered.

UNDERWATER HARVESTER'S ASSOCIATION GEODUCK ENHANCEMENT PROGRAM. Bruce Clapp, Bruce Clapp Biological Consulting, Ladysmith, B. C., V0R 2E0.

The Underwater Harvester's Association (UHA) is comprised of all the licensed geoduck and horseclam fishermen in British Columbia (BC). The geoduck fishery started in BC in 1976, the UHA was formed in 1981 and in 1989 the fishery changed to an Individual Vessel Quota (IVQ) system, which was initiated by the UHA. From 1989 to 1998 the UHA saw a continual reduction in their annual quota. The reduction in quota was a result of changes in the knowledge of bed areas and geoduck density estimates. In 1994 the UHA funded and initiated an enhancement program to plant hatchery raised juvenile geoducks in existing beds to enhance the local populations. The initial objectives for the program were to explore the feasibility of geoduck enhancement, to increase stock for brood and eventually to offset harvest (partially). This was the first shellfish fishery to try enhancement in BC. There were no hatchery facilities in BC and no planting technology available for the UHA to copy. In 1999, there was a successful geoduck hatchery and the UHA has a machine that can plant up to 50,000 juvenile geoducks per day. The details of how this enhancement program would be incorporated into the wild geoduck management plans have not been explored.

CO-MANAGEMENT AND ASSESSMENT PROGRAMS IN THE SHRIMP TRAWL FISHERY OF BRITISH COLUMBIA. Rick Harbo and **L. Convey,** Fisheries and Oceans Canada, Operations Branch, Fisheries Management, Pacific Region, 3225 Stephenson Point Road, Nanaimo, B.C. V9T 1K3; **J. A. Boutillier,** Fisheries and Oceans Canada, Science Branch, Pacific Region, Pacific Biological Station, 3190 Hammond Bay Rd., Nanaimo, B.C. V9R 5K6.

The diverse and complex Pacific shrimp trawl fishery takes place along the British Columbia coastline, in a number of small inshore areas and large offshore grounds. The fleet of 248 licences is a mix of beam and otter trawls. There are seven Pandalid species harvested commercially and fisheries vary in complexity from single to multiple species fisheries with a variety of markets, including machine-peeled, hand-peeled, frozen-at-sea, fresh and live shrimp.

Landings peaked at over 7,300 tonnes, with annual landed values reaching $Cdn 13.6 million. Landings have declined since 1996, to annual levels ranging from 2,000 to 3,000 t @ $Cdn 5 to 7 million, due to low stock levels in offshore areas and more restrictive, precautionary management practices.

In response to a dramatic increase in effort on this fishery, management has developed rapidly from passive management at relatively low levels of efforts to a complex suite of management programs starting in 1997. The offshore pink shrimp fishery on the west coast of Vancouver Island is managed by a seasonal opening. For the inshore fisheries and the remaining offshore areas, fixed arbitrary, historically based or forecast catch ceilings (TACs) are initially assigned to more than 30 individual Shrimp Management Areas. These initial catch ceilings are adjusted in-season when information from the fishery or a biomass survey indicate the area can sustain fishing pressure either less than or greater than the initial levels.

Development towards a biologically based management strategy is ongoing. A long-term collaborative management and assessment program has been initiated with stakeholders that includes catch monitoring, catch sampling, biological sampling, and fishery independent surveys. An index system of assessment is being combined with an experimental management approach to develop the critical data necessary to model and manage this diverse and complex set of shrimp fisheries.

AN ECONOMIC ANALYSIS OF THE GULF OF MEXICO OYSTER PROCESSING SECTOR. Assane Diagne,* Louisiana Department of Wildlife and Fisheries (LDWF)—Socio-Economic Section, 2000 Quail Dr. P.O. Box 98000, Baton Rouge, LA 70898-9000; **Walter R. Keithly, Jr.,** Center for Coastal, Energy, and Environmental Resources, Louisiana State University, Baton Rouge, LA 70803; **David Lavergne** (LDWF).

Annual oyster landings in the Gulf of Mexico account for approximately 60 percent of annual landings in the United States. Over the past five years, annual oyster landings in the Gulf aver-

aged 26 million pounds, valued at $40 million. Along with other seafood species harvested, this sizeable supply of fresh oysters helps support the dynamic seafood industry that has developed in the Gulf and throughout the southeastern United States. In 1997, the oyster processing sector generated in excess of $60 million. This study evaluates the market structure of the oyster processing industry in the Gulf of Mexico. Structural parameters used to analyze the oyster processing industry include the number of dealers operating in the industry, their size distribution, degree of diversification, and the concentration in the industry as measured by several concentration indices. Calculated structural parameters are used to draw economic inferences on market conduct and to make comparisons between the different states.

MANAGEMENT STRATEGIES FOR COMMERCIAL INTERTIDAL CLAM FISHERIES IN BRITISH COLUMBIA, CANADA. Rick Harbo[1] and **Randy Webb,** Fisheries and Oceans Canada, 3225 Stephenson Point Road, Nanaimo, B.C. V9T 1K3 Canada.[1]

There are a number of intertidal clam fisheries in B.C. including First Nations fisheries for food, social and ceremonial purposes, commercial fisheries and recreational fisheries managed by the federal Department of Fisheries and Oceans. The four commercially harvested species (landings in 1998) are Manila clams, *Venerupis philippinarum* (1,115 tonnes), native littleneck clams *Protothaca staminea* (50 t), mixed (118 t), butter clams *Saxidomus gigantea* (40 t) and razor clams *Siliqua patula* (40 t).

The commercial fishery has historically been managed by minimum size limits and time and area closures. Fisheries are monitored in-season against historical landings and effort, and once these ceilings are reached in any given fishing area, the fishery may be closed. Extensive consultation takes place and an effort to develop Community Management Boards has been initiated in two areas. The fisheries are designed to allow openings throughout the year in order to deal with market demands. Area management (1989) divided the coast into 7 areas. Licence limitation (1998) reduced the number of harvesters from approximately 2000 to a fixed number of 1160. Approximately 50% of these licence holders are First Nations participants. First Nations also participate in the co-management of beaches fronting or immediately adjacent to Reserves and pilot projects in the north coast.

Harvests for depuration are managed experimentally by quotas, setting a variety of exploitation rates (0 to 50%). A collaborative agreement was developed with industry that supports surveys and stock assessment programs and a fishery manager. Five plants are licensed to depurate in B.C.; harvesting >400 t in 1998 from vacant crown foreshore. Additional harvests from aquaculture leases in open and contaminated areas are managed by the province.

THE COMMERCIAL GEODUCK (*PANOPEA ABRUPTA*) FISHERY IN BRITISH COLUMBIA, CANADA—AN OPERATIONAL PERSPECTIVE OF A LIMITED ENTRY FISHERY WITH INDIVIDUAL QUOTAS. Stephen Heizer, Fisheries and Oceans Canada, 3225 Stephenson Point Road, Nanaimo, B.C., Cda., V9T 1K3.

The geoduck fishery in British Columbia began in 1976 as an open access fishery without catch limits. In 1979, entry to the fishery was limited to 55 licences and total allowable catches were introduced. In 1989, equal individual vessel quotas were introduced, at industry request, in response to excessive effort and concerns over product supply and handling, safety and conservation. Subsequently, the fishery became BC's most valuable invertebrate fishery, worth approximately CDN$40 million annual landed value and supplying a high quality live product year round.

The IQ fishery has resulted in improved fishery management and assessment. An industry-funded dockside monitoring program improved the timeliness and quality of reporting of catch and effort data. Quota overages have been limited to less than 0.1% annually compared to overages of 55% in pre-IQ periods. Improved tracking of product has aided enforcement. The geoduck fisher's association provides over CDN$1 million annually towards the management and assessment of the fishery. Funds cover water quality certifications, biotoxin monitoring, funding for a fishery manager, fisheries research and stock assessment activities.

More biological research is being done with the support of industry vessels and divers, and managers have greater operational flexibility to deal with real-time biological, enforcement and logistic issues. Health and safety in the industry has improved greatly. Diver deaths and injuries and vessel accidents have decreased. Fishing plans are developed for two-year terms, and co-management agreements for periods up to 5 years. Industry is seeking longer and guaranteed terms of access.

RIDING THE ROLLERCOASTER: BOOM AND DECLINE IN THE CALIFORNIA RED SEA URCHIN FISHERY. Peter E. Kalvass,* California Department of Fish and Game, 19160 S. Harbor Dr., Fort Bragg, CA 95437.

Initiated as an experimental fishery in southern California in 1971, the red sea urchin catch reached over 10 million pounds in 1981 prior to a three year El Nino related decline. Rapid growth of the fishery into northern California between 1985 and 1987 fueled concern that specific management measures were needed as permit numbers and catch more than doubled, to 915 permits and nearly 50 million pounds. As a result, the legislature enacted additional landing taxes to fund gathering of fishery data, research on population parameters and resource enhancement, and the creation of an

industry advisory committee to assist the Department in developing further management measures. The state Fish and Game Commission adopted a formal limited entry system with a target number of fishery participants and the first minimum size requirements in 1989. Separate harvesting closure periods in northern and southern California were established in 1990. While it was the policy of the Commission to give consideration to maximum sustained yield in its management of marine resources, there were no scientific management mandates prior to the adoption of the Marine Life Management Act in 1998. Consequently, management followed a reactive 'points of concern' approach designed primarily to reduce harvesting pressure in the face of evidence that recruitment overfishing has occurred in northern California, and that the harvestable stock has been serially depleted in southern California. By 1998, following two El Ninos in the 1990s and a weakened Japanese export market for uni, the statewide catch had steadily declined to 10.5 million pounds valued at $8 million, from a high in 1988 of 52 million pounds, worth $35 million.

TEMPORAL AND SPATIAL VARIATION IN SPAWNING OYSTER (*TIOSTREA CHILENSIS*) DISTRIBUTION IN FOVEAUX STRAIT, NEW ZEALAND. **Jonathan A. Keogh,*** Portobello Marine Laboratory, University of Otago, P O Box 8, Portobello, New Zealand; **David J. Fletcher,** Centre for the Application of Statistics and Mathematics, University of Otago, P O Box 56, Dunedin, New Zealand.

A stratified random stock assessment survey using a standard double-sided oyster dredge was conducted over a three week period in October of 1999 throughout the Foveaux Strait oyster fishery in southern New Zealand. As this timing was co-incident with the austral spawning season and since the oysters surveyed brood their larvae up to the late pedi-veliger stage, legal sized oysters (>58 mm shell length) from the 210 stations sampled were retained, landed and opened so as to assess spawning oyster occurrence. For each station estimates were made of the number of non-brooding oysters and the number of oysters bearing eyed or non-eyed larvae, the brood size and oyster condition. The relationship between the number of spawning oysters at each station and oyster density and size distribution is reported on and the implications of this for larval fishing as a source of larvae for stock enhancement is discussed.

UNCOVERING BENEFITS OF WEST INDIAN CROWN CONCH OR 'CHIVITA' (*MELONGENA MELONGENA*) IN YUCATÁN, MEXICO. **Michael D. Kaplowitz,*** Department of Resource Development, Michigan State University, East Lansing, MI 48824.

Focus groups and individual interviews were conducted with local resource beneficiaries as part of the design phase for an economic valuation study of mangrove ecosystems of Yucatán, Mexico. The research examined how local resource beneficiaries use, perceive of, and understand the ecological services associated with their shared mangrove ecosystem. The data revealed that collection of West Indian Conch (*Melongena melongena*) locally called chivita has become an increasing part of the communities' economic activity. Surprisingly, chivita collection appears to have replaced other resource-based subsistence strategies in these communities. These findings place *Melongena melongena* in a new light since heretofore both marine resource beneficiaries and shellfish researchers have viewed this species as little more than a predator species with little or no benefits. The reported research also uses data collected from local beneficiaries to estimate economic benefits of chivita collection to these communities. The analysis demonstrates that the current extractive use benefits to these communities of *Melongena melongena* for subsistence and commercial sale are significant. Furthermore, the research reveals conflicting management agendas for the shared mangrove ecosystem including some that threaten the continued viability of chivita collection.

CLAM FISHERY IN BRAZIL. **Carla Medeiros y Araújo,*** Universidade de Brasília-IB-GEM; **Iara L. G. Brasileiro,** Universidade de Brasília—Centro de Desenvolvimento Sustentável SAS Qd.5, B1.H, 2° andar, Brasília-DF-Brasil.

A lot of bivalve species are easy to collect and are largely consumed by coastal populations. Since 1920 some researchers (Edmondson, 1920; Quayle, 1943, Eldridge *et al*, 1979) have been worrying about this situation and nowadays sustainable development politics is linking conservation and exploitation. Brown mussel *Perna perna* cultures and clam *Anomalocardia brasiliana* management fishery in South of Brazil are examples of new political strategies. *Dosinia concentrica, Lucina pectinata, Trachycardium muricatum, Iphigenia brasiliana* and *Prothothaca pectorina* are yet consumed without any fishery management control in northeast and south regions of Brazil. The aim of Sustainable Development Centre is to obtain more data about fishermen communities in social, health and economical aspects. A number of specimens were collected in Florianópolis (27°38′SE, 48°33′05″W) (Santa Catarina) and Maragojipe (12°33′S, 38°00′W) (Bahia) in order to make preliminary studies. Both places show no controlled fishery and few biological information. But in Bahia, clam consumption is more intensively allied to precarious commercial structure and health control. Improved biological information about the Brazilian clam fishery is needed. This will lead us to better comprehension of the social situation of fishermen in our community.

CHESAPEAKE BAY OYSTERS: TRENDS IN RELATIVE ABUNDANCE AND BIOMASS. **Carol McCollough, Stephen J. Jordan,*** and **Mark L. Homer,** Maryland Department of Natural Resources, Sarbanes Cooperative Oxford Laboratory, 904 S. Morris St., Oxford MD 21654.

Oyster populations are distributed patchily over more than 400,000 acres in Chesapeake Bay, so it is not feasible to assess their absolute numbers or biomass. Traditionally, landings data, with their inherent inaccuracies and biases, have been the only consistent means of estimating trends. A long term monitoring program in Maryland has recorded relative numbers and size distributions of oysters, along with other population and disease data annually; 43 fixed sites have been monitored consistently since 1990, with many records from these sites available from earlier years. In 1999, we obtained shell height measurements and dry tissue weights from samples of 10 oysters from each site (selected to represent the range of sizes present). By applying the resulting length:weight equation to size-frequency data from earlier surveys, we computed an index of relative biomass that varied from year to year according to the relative abundance and size distribution of the oyster populations. The index is useful for portraying trends and tracking the performance of restoration efforts. It reflects interannual variations in recruitment and growth, as well as mortality caused by the oyster parasites *Haplosporidium nelsoni* and *Perkinsus marinus*.

MANAGEMENT OF THE CANADIAN PACIFIC COAST PRAWN TRAP FISHERY: RECENT CHANGES, PRESENT STATUS AND FUTURE OPTIONS. **Jim Morrison,** Fisheries and Oceans Canada, 3225 Stephenson Point Road, Nanaimo, B.C., Cda., V9T 1K3.

The spot prawn *Pandalus platyceros* is the largest of seven commercial pandalid shrimp species occurring in B.C. waters. In 1997 this was the sixth most valuable fishery on Canada's Pacific coast, valued in excess of $30 M. (Cdn). It is a competitive fishery limited to 253 licence holders, with vessel length limits, gear specifications and size limits. Japan is the largest market for frozen at sea product.

Recent significant changes in this fishery began with the adoption of trap limits in 1995, followed by increases in effort due to external factors as well as changes in fishing behaviour. This resulted in recent record landings and record values while the fishing season declined in the last 6 years from 230 days to 79 days. Simultaneously, stock protection targets have been achieved due to in-season biological sampling provided by 11 industry funded charter patrol vessels and the fishery is being managed in a more conservative manner than historically. However, fishermen have expressed concerns for the fishery.

Future management options are described based on a discussion paper written by elected industry representatives of the advisory committee. A pilot program will be implemented in 2000 to test and compare two management regimes, one with single gear pulls per day intended to reduce catches of undersized prawns, and one which would continue the present intense multiple haul fishing pattern.

Planning for the 2001 fishery remains uncertain due to changing DFO policies regarding industry funding for fishery management. Present funding arrangements will sunset in March 2001. Options for "partnering" with industry for co-management and co-funding will be described.

MANAGEMENT OF THE DUNGENESE CRAB FISHERY IN BRITISH COLUMBIA. **Guy Parker,** Fisheries and Oceans Canada, 3225 Stephenson Point Road, Nanaimo, B.C., Canada, V9T 1K3; **Kim West,** Fisheries and Oceans Canada, 610 Derwent Way, New Westminster, B.C., Canada, V3L 5B3; **Ivan Winther,** Fisheries and Oceans Canada, 417-2nd Ave West, Prince Rupert, B.C., Canada, V8J 1G8.

Dungeness crabs are harvested extensively throughout British Columbia by First Nations, recreational, and commercial fishers. The commercial fishery began back in the 1880's, and has expanded to become the seventh most valuable wild fishery in British Columbia during 1997, worth an estimated $28.7 million. Landings in 1997 reached approximately 3,000 tonnes, slightly lower than the average annual landings of 4,000 tonnes that have been commercially harvested during the 1990's.

Until the late 1980's much of the management and conservation measures within the crab fishery were passive, relying on a minimum size limit for harvest, non-retention of females, and a few small time and area closures. Increases in the number of participants in the fishery and increases in over-all fishing effort have led to the need for more active management, including licence limitation, area licencing, harvest logbook requirements, vessel trap limits, and an increasing number of time and area closures.

SCIENCE AND MANAGEMENT OF GREEN SEA URCHINS IN BRITISH COLUMBIA—A REBUILDING FISHERY? **R. Ian Perry,*** Fisheries & Oceans Canada, Pacific Biological Station, Nanaimo, B.C. V9R 5K6, Canada; **Guy Parker,** Fisheries & Oceans Canada, 3225 Stephenson Point Rd, Nanaimo, B.C. V9T 1K3, Canada; **Juanita Rogers,** Fisheries and Oceans Canada, 417 2nd Ave. W, Prince Rupert, B.C. V8J 1G8, Canada.

The green sea urchin (*Strongylocentrotus droebachiensis*) fishery in British Columbia is a small but important component of the province's dive fisheries. It has had a typical boom and bust profile of a developing fishery, reaching peak landings of 978 tonnes (value Cdn$4.5 million) in 1992, then declining to about 150 tonnes in 1995. These declines were driven in part by increasingly restrictive management regulations. Since 1995, stock assessment activities have been conducted annually, including surplus produc-

tion estimates of sustainable yields and fishery-independent surveys (conducted jointly with industry) of abundance, size composition, and recruitment. Resulting management actions included restricting the fishery to two core fishing areas, minimum size limits, and area and individual quotas. Since 1995, landings have stabilized and the catch per unit of effort has been increasing. A program of re-opening areas to fishing has been established which requires surveys to "prove the resource", which the industry has keenly adopted and is actively pursuing. The green urchin fishery in B.C. appears to be rebuilding, although not at the same rate in both core areas.

MANAGEMENT OF THE RED SEA URCHIN FISHERY IN BRITISH COLUMBIA. Juanita Rogers and **Guy Parker,** Fisheries and Oceans Canada, Pacific Region.

The Red Sea Urchin fishery began in 1971 and expanded quickly until 1992 when landings reached nearly 13,000 tonnes. Today, there are 110 personal licences eligible to fish Red Sea Urchins annually, with a total commercial allowable catch of approximately 5,600 tonnes. The value of the fishery is susceptible to the Japanese markets and economy, with the average annual price fluctuating between $1,300 and $2,000 per tonne since the implementation of an individual quota (IQ) program in 1994.

Management in the red urchin fishery has become increasingly more active. Current management measures include a minimum size limit of 100 mm, licence limitation and area licensing, area quotas calculated using a fixed exploitation rate of area biomass estimates, individual licence quotas, catch validation and monitoring, and fishery-independent biomass surveys.

The Pacific Urchin Harvesters Association (PUHA) represents all licence holders, is responsible for developing the catch validation program, and is a major contributor to research surveys, both through direct funding and in-kind support.

COUPLING A COMPUTATIONAL FLUID DYNAMIC MODEL WITH A HABITAT SUITABILITY INDEX MODEL TO MANAGE EASTERN OYSTERS IN MOBILE BAY, ALABAMA. Leonard J. Rodgers and **David B. Rouse,** Department of Fisheries and Allied Aquacultures, Auburn University, Auburn, AL 36849.

The need to quantify and qualify habitat is an important component of resource management. Habitat suitability index (HSI) models are used to spatially delineate essential habitat. When considering the implementation of an HSI for Eastern oysters (*Crassostrea virginica*) in an estuarine environment, an investigator estimates highly dynamic spatial variables such as temperature, salinity, and hydraulic flux.

In general, extremely simplified methods of estimating spatial variables are incorporated into HSI models of estuarine systems. One example would be the use of mean monthly water parameters from a few fixed sites to characterize an estuary. The current study uses a two dimensional computational fluid dynamic (CFD) model to calculate a measure of central tendency and a variance for major hydrodynamic variables of oyster habitat on a tessellated grid. Spatial data from several sources were combined with the CFD to determine suitability indices. Using the U.S. Fish and Wildlife Service geometric mean method an HSI was calculated. Methods, applications, implications and limitations of this modeling method will be discussed.

THE PRICE OF SUSTAINABILITY IS ETERNAL VIGILANCE. Scoresby A. Shepherd, South Australian Research and Development Institute, PO Box 120 Henley Beach 5022, South Australia.

The South Australian abalone fishery has been going for about 34 years. There have been some declines, but the majority of stocks of blacklip, Haliotis rubra, and greenlip *H. laevigata*, have been sustained under fishing. The input and output measures used to control fishing are described. However, much is to be learned from the study of stocks which have declined. Three stocks of greenlip abalone were monitored by survey up till the point of collapse. They have provided remarkable insight into the effect of fishing on population processes. Three consequences are set out below.

1. Because divers fish for aggregations they are able to reduce fertile gamete production much more that is indicated just by the reduction in density. This is the Allee effect.

2. Abalone metapopulations are spatially structured and often occupy habitats that are differentially vulnerable to fishing. Stock-recruitment curves for vulnerable and resilient habitats within a metapopulation indicate that higher densities must be retained in the former than in the latter habitats to avoid spatial contraction (and ultimately collapse) of the stock.

3. At a larger spatial scale, small metapopulations are more vulnerable to overfishing that larger ones. The first implication from this is that serial depletion is certain unless management measures are tailored to ensure the persistence of the most vulnerable populations. Second, if even mild overfishing of large populations occurs, they will slide downhill as productivity declines and become more vulnerable to overfishing.

These consequences indicate that spatial measures such as rolling closures and refugia are valuable for management of multiple sedentary stocks with limited larval dispersal in addition to other traditional measures such as quotas, and size limits.

Given the usual fuzzy understanding of the status of individual abalone stocks the use of an ensemble of fishery indicators in a "trouble spot thermostat" approach (inspired by Caddy 1999) is proposed. As the temperature rises in a stock and indicators are sequentially triggered increasingly severe management responses are generated, until at the extreme the area is closed. A system devised for the South Australian abalone fishery is outlined.

MANAGEMENT OF THE PACIFIC RAZOR CLAM AND THE CURRENT MARINE TOXIN THREAT IN WASHINGTON STATE. **Doug Simons** and **Dan L. Ayres**, Washington State Department of Fish and Wildlife, 48 Devonshire Rd., Montesano, WA 98563.

The Pacific razor clam *Siliqua patula*, (Dixon) has been closely managed and monitored in Washington State since the early 1900's. What began as a large and important commercial fishery, evolved into a major recreational shellfish fishery. Upwards of 15 million clams were harvested in the recreational fishery during the peak years in the late 70's by almost one million digger trips. This magnitude of usage prompted Washington State to manage the resource in a continually increasing conservative manner. Seasons were shortened and limits lowered repeatedly when population and catch levels continued to decline. Since the mid 70's, the resource has gone through more major turmoil than all the years leading up to then. In the late 70's massive wastage of small clams occurred due to a disproportionate recruitment in the population. In the 80's, a specie specific disease caused the loss of over 90% of all razor clams in Washington State. In 1991, a new marine toxin was discovered that totally changed the way razor clams are now managed and in 1993, coastal Indian Tribes began exercising their Federally recognized treaty rights to harvest razor clams off reservation. This paper briefly summarizes the major transitions in management with the focus on the current influence of marine toxins and tribal co-management.

MANAGEMENT OF GEODUCK CLAMS (*PANOPEA ABRUPTA*) IN WASHINGTON STATE. **Bob Sizemore,*** Washington Department of Fish and Wildlife, Point Whitney Shellfish Laboratory, Brinnon, WA 98320.

Washington's commercial geoduck clam fishery began in 1970. Annual subtidal SCUBA dive surveys began in 1967. Population size is based on surveyed harvest tracts located between the −5.5 meter (MLLW) and −21.3 meter water depth contours. Annual landings peaked at 3901 t in 1977, followed by a restricted total allowable catch (TAC) of 2268 t from 1979 to 1988, 1497 t from 1989 to 1995, and a range of 1678 t to 1950 t from 1996 through 1999. The average annual ex-vessel value of the commercial geoduck fishery in Washington state is US$14 million for years 1990 to 1998. Prior to 1997, geoduck yield for the commercial subtidal fishery was based on a Ricker yield per recruit model. In 1997, managers adopted a deterministic, age-structured equilibrium yield model and recommended a TAC of 2.7% of the commercially available biomass in six management regions. The rate of recovery on harvested tracts is empirically evaluated by a series of post-harvest SCUBA surveys on 15 tracts scattered throughout Puget Sound. A tagging study, at one location, has been completed to identify methods to empirically verify the yield model mortality parameter. Poaching and high-grading have led to under-reporting, overharvest, and wastage of the geoduck clam resource.

RESEARCH ACTIVITIES IN SUPPORT OF ALASKAN CRAB FISHERIES. **Bradley G. Stevens,** National marine Fisheries Service, Kodiak Fisheries Research Center, 301 Research Ct. Kodiak, Alaska, 99615.

Both the National Marine Fisheries Service (NMFS) and the Alaska Dept. of Fish and Game (ADF&G) conduct research in support of Alaskan crab fisheries. Research activities fall into four general categories: (1) Studies to improve survey methodology include estimating catch efficiency of survey trawls, effects of variable tow length, adaptive sampling, and alternative techniques such as video and laser scanning devices. (2) Maintenance of healthy stocks and reproductive capacity requires research on mating behavior, size at maturity, fecundity, terminal molt, mortality factors, shell aging, and genetic stock discrimination. (3) Understanding and preventing harmful effects of fishing requires studies of pot loss, ghost fishing, discard mortality, escapement behavior and devices to allow escapement, pot design and functionality, and improvement of species recognition. (4) Additional research is targeted towards understanding basic biology of crabs and long term recruitment trends, and includes studies on reproductive development, hatching behavior, culture techniques and larval survival, settlement and habitat use, endocrinology, studies of aggregation and burial behavior, tagging, and environmental effects on reproduction and recruitment.

DUNGENESS CRAB (*CANCER MAGISTER*) MANAGEMENT IN PUGET SOUND, WASHINGTON. **Derrick R. Toba,*** The Tulalip Tribes, Tulalip Shellfish Program, 7615 Totem Beach Road, Marysville, WA 98271.

Dungeness crab (*Cancer magister*) is one of the most important commercial and recreational shellfish species in Washington State. In 1994, the federal district court ruled that the Western Washington Treaty Tribes reserved the right to harvest shellfish under the Treaties. Following prior rulings by Judge Boldt in 1974, the Tribes had a right to harvest up to 50% of the sustainable harvest biomass of shellfish in their usual and accustomed fishing areas. As a result of this decision, the crab resource is cooperatively managed by the State of Washington and affected Treaty Tribes in a given region. The management of Dungeness crab for the Puget Sound and Strait of Georgia area is divided into six management areas.

The management of Dungeness crab is similar throughout the West Coast of North America. One of the current management goals is geared toward protecting soft-crab and is based on historical studies that were conducted by Washington Department of Fisheries. The State of Washington has traditionally used June 1 through July 15 as a time period to close its fishery to protect soft-shell crab. Since the data was collected primarily from the northern portion of Puget Sound, this closure did not fit all management areas.

Additional biological information is being collected in various regions to determine deviations from the traditional closure period. This includes crab hardness tests for legal sized male crab, condition of females and sub-legal male crab. In addition, other indicators may be used to determine the timing of the molt and closure of the fishery. The studies indicate that there are variations within regions and that crab management needs to conform with the biological requirements of the region.

CALIFORNIA ABALONE FISHERIES: WHAT WE'VE LEARNED AND WHERE WE GO FROM HERE. Mia J. Tegner, Scripps Institution of Oceanography, University of California, San Diego, La Jolla, CA 92093-0201.

California once supported fisheries for five species of abalones (*Haliotis* spp.). Found in predictable and accessible locations near stands of their algal food, these animals exhibit irregular recruitment, grow slowly, and, as adults, move very little. They are preyed on by sea otters and a host of other predators; fishable stock sizes resulted from the extirpation of otters. Human fishing, which dates to 9,775 years bp, has an extensive history. Modern Southern California fisheries began in the 1940s, remained high until the late 1960s, and then began a precipitous decline; all commercial and sport fishing south of San Francisco was halted in 1997. Limited entry to the commercial fishery and experimental enhancement programs instituted in the 1970s failed to stem the decline. Management was based largely on size limits that should have allowed adequate egg production; stock assessments were rare and environmental variability was not taken into account. The importance of maintaining patches of spawners at densities adequate to support fertilization was learned only after many local populations collapsed to densities so low that remaining animals are functionally sterile. High economic value driving intense search efforts, the sea urchin fishery, gear improvements, El Niño events affecting feeding and reproduction, and abalone disease all contributed to the decline. Today, white (*H. sorenseni*) and black (*H. cracherodii*) abalones are under evaluation for the Endangered Species List. In contrast with Southern California, the coast north of San Francisco has been reserved for sport fishing since 1945 and gear restrictions prohibit the use of compressed air. This fishery has been sustained at high levels despite extensive poaching pressure; the *de facto* depth reserve has apparently maintained brood stocks and recruitment. Recovery efforts in Southern California are aimed at aggregating rare species into enforced closed areas to protect brood stocks and allow for natural reproduction. The conundrum of sea otters, now foraging below Point Conception, remains to be resolved.

BIOLOGY AND MANAGEMENT OF EASTERN BERING SEA KING AND TANNER CRAB FISHERIES. Donn A. Tracy, Alaska Department of Fish and Game, Commercial Fisheries Division, Kodiak, AK 99615; **Brad Stevens,** National Marine Fisheries Service, Kodiak Fisheries Research Center, Kodiak, AK 99615.

Commercial king and Tanner crab fisheries of the Eastern Bering Sea are regulated under the joint jurisdiction of the National Marine Fisheries Service and the Alaska Department of Fish and Game. Fishery management goals are accomplished by incorporating the 3-S principle (size-sex-season) into harvest strategies based upon estimates of mature biomass, exploitation rates applied to mature males and minimum abundance thresholds. Stock assessment methods include trawl surveys from which area-swept estimates of abundance are derived (in some fisheries length-based-assessment models are applied to annual area swept estimates to minimize survey measurement error). Adoption of harvest strategies into fishery regulations create a foundation for implementation of shorter term management measures (e.g., pot limits, reporting requirements) necessary to attain overall conservation objectives. Application of state and federal management policy (including remedial plans for jeopardized stocks) are illustrated by examining two high profile fisheries: Bristol Bay red king crab and EBS Tanner crab.

LARVAL RECRUITMENT OF *MYA ARENARIA* (SOFT-SHELL CLAMS) IN EASTERN AND SOUTHERN MAINE. Tracy Vassilieν* and **William Congleton,** Department of Biosystems Science and Engineering, University of Maine, Orono, ME 04469; **Brian Beal,** University of Maine Machias, Machias, ME 04654; **Stephen Fegley,** Maine Maritime Academy, Castine, ME 04420.

Clam landings have dramatically decreased in Eastern Maine since 1982. Densities of 0-year *M. arenaria* recruits were sampled in two Maine counties, Washington County (Downeast) and Cumberland County (Southwest), by taking intertidal core samples (0.0133 m^2) over three years (1996–1999). Each year Washington County had significantly fewer *M. arenaria* recruits than Cumberland County ($P < 0.001$). To determine whether this recruitment difference was due to lack of *M. arenaria* larvae in near-shore waters or post-settlement mortality, spat bags filled with monofilament were placed in Mason Bay (Eastern Maine) and the Scarborough River (Southern Maine), during the summer of 1998 (May–October). The spat bags were replaced monthly and contents sieved through a 750 μ screen. The Scarborough River had sig-

nificantly more *M. arenaria* per spat bag during the mid summer months ($P < 0.001$) suggesting Eastern Maine's lack of *M. arenaria* recruits is due to reduced densities of larvae in near-shore waters rather than post-settlement mortality or location rejection. These results indicate increased juvenile recruitment could enhance the soft-shell clam fishery in Eastern Maine.

BUT THE CRABS KEEP COMING: TRIALS AND SUCCESSES OF THE GRAYS HARBOR DUNGENESS CRAB MITIGATION PROGRAM. Lauran Cole Warner,* Seattle District Corps of Engineers, PO Box 3755, Seattle, WA 98124; **Eileen P. Visser,** 14 Spring Street, Potsdam, NY 13676.

The Corps of Engineers built and maintains a navigation channel from the mouth of the Grays Harbor estuary up to ports near Aberdeen, Washington. That navigation channel was widened and deepened beginning in 1990 so that bigger ships could call these ports. Environmental studies leading up to this project found that Dungeness crabs, *Cancer magister,* are entrained and killed during dredging. Mitigation for this loss consists of placing aged oyster shell on the intertidal mud flats of Grays Harbor, providing habitat for young-of-the-year *C. magister* that otherwise would not survive. Since the first test plots were built in 1990, over 60 hectares of oyster shell have been placed in the Grays Harbor intertidal area, producing over 12 million juvenile crab. But there have been many obstacles along the way: oyster shell disappeared under the mud much more quickly than optimistic planners had hoped for; green shore crab (*Hemigrapsus oregonensis*) overtook plots after the first year, staying for the winter and apparently outcompeting settling Dungeness the following summer. In addition, crab impacts continued to pile up as the channel was maintained each year, and the mitigation program fell deeply into debt. Changes in both the mitigation and dredging programs have since been made to both reduce impacts and increase the efficiency of mitigation.

STOCK ASSESSMENT OF SURFCLAMS ALONG THE EAST COAST OF THE UNITED STATES: THE IMPORTANCE OF ESTIMATING DREDGE EFFICIENCY. James Weinberg, Paul Rago, Charles Keith, Lisa Hendrickson, and **Steve Murawski,** NMFS, Woods Hole, MA 02543; **Eric Powell,** Haskin Shellfish Lab., Rutgers University, Port Norris, NJ 08349; **Roger Mann,** College of William and Mary, VIMS, Gloucester Pnt., VA 23062; **Chris Weidman,** WHOI, Woods Hole, MA 02543.

The National Marine Fisheries Service (NMFS) has conducted surveys of Atlantic surfclam (*Spisula solidissima*) populations along the USA Atlantic coast since 1980 with a standardized hydraulic clam dredge. To estimate the absolute abundance of clams in an area for stock assessment, the efficiency, *E*, (i.e., probability of clam capture given encounter with the gear) of the survey dredge must be known. To compute *E* for the dredge on the NMFS research vessel (*R/V Delaware II*), we performed five field experiments in 1997 with the *R/V Delaware II*, and three commercial clam vessels. We compared a swept area catch estimate from the *R/V Delaware II* with density estimates from depletion experiments conducted by commercial vessels at the same sites. The data were analyzed using an extension of the traditional theory for analyzing depletion experiments. For each tow in the depletion experiment the expected catch was modeled as a chain binomial process whose parameters are a function of catches in previous tows at that site. The model also takes into account the degree of spatial overlap among tows. Estimates of *E* for the NMFS survey dredge ranged from 0.23–0.46. Results from these experiments were used in recent surfclam stock assessments. Additional experiments are being planned because *E* is likely to vary with depth, sediment type and sea state.

MHACS: MARINE HABITAT ACOUSTIC CHARACTERIZATION SYSTEMS. A PROGRAM FOR THE ACQUISITION AND INTERPRETATION OF DIGITAL ACOUSTICS TO CHARACTERIZE MARINE HABITAT. Charles A. Wilson and **Harry H. Roberts,** Coastal Fisheries and Coastal Studies Institutes, Department of Oceanography and Coastal Sciences, CCEER; **John Supan,** Office of Sea Grant Development, Louisiana State University Baton Rouge, LA 70803.

Coastal Louisiana, like many deltaic land-masses, faces continued landscape alteration from natural processes and anthropogenic impacts that affect fisheries production. Many steps are being taken at both State and Federal levels to slow/mitigate these changes. Most promising of these strategies is river diversions, which introduce freshwater and sediment to river-flanking environments (lakes, bays, and associated marshlands). Two such diversion projects, planned by Louisiana Department of Wildlife and Fisheries and U.S Army Corps of Engineers (Caenarvon and Davis Pond), are designed to nourish marshes with water and sediment as well as to help establish ideal isohalines over historic oyster grounds. Critical to the success of these programs is a rapid and accurate means to qualify and quantify changes in marine habitat in the Barataria Basin. Digital high resolution acoustic instrumentation linked to state-of-the-art data acquisition and processing software is available for building a baseline of information that can be used for evaluating future changes in shallow and shelf water bottoms with special emphasis on fisheries habitat.

Application of dual beam hydroacoustics (120 khZ), digital side-scan sonar (100 and 500 kHz), a broad-spectrum sub-bottom profiler (4–24 kHz) for rapidly acquiring water column, surficial and shallow subsurface data has now been accomplished. These data sets, "calibrated" with trawling, surface sampling, coring, and

other "ground-truthing" techniques, have enormous potential for understanding (a) distributions of bottom sediment types (including man-made objects), (b) locations of oyster reefs and distributions of scattered oyster clumps and shells, (c) fisheries habitats, (d) areas of active sedimentation and erosion, and (e) shallow subsurface configurations that influence surface conditions. In our most recent study, geo-referenced side-scan sonar mosaics of surveyed oyster lease areas were incorporated into a GIS database. Using image-processing techniques to analyze mosaic reflectance patterns, we estimated the percent and total acreage of several bottom types. Results were calibrated with field collected ground truth measurements.

MANAGING SHELLFISH CULTURE IN COASTAL WATERS

ENDANGERED SPECIES ACT AND SUSTAINABLE FISHERIES ACT IMPLICATIONS FOR MOLLUSCAN SHELLFISH CULTURE MANAGEMENT. William F. Dewey, Taylor Shellfish Company, Inc., 130 SE Lynch Road, Shelton, WA 98584.

The Endangered Species Act and the Sustainable Fisheries Act are two federal laws with broad reaching powers. These Acts with their sweeping ability to protect species and critical or essential habitat required by the species at all costs, have the potential to drastically alter estuarine management strategies with little regard for the economic impact on the shellfish culture industry. Triggered by the federal nexus of Army Corp permits or by receiving federal research dollars, growers find themselves facing a whole new layer of intimidating federal bureaucracy never before experienced. Several West Coast estuaries now have wild runs of salmon listed as threatened or endangered under the Endangered Species Act. Eleven species of Puget Sound bottom fish are currently being considered by the National Marine Fisheries Service for listing. Section 9 of the ESA prohibits "take" of threatened or endangered species. A "take" not only constitutes killing the species but includes any activity that hurts or harms any aspect of the species' lifecycle, including damaging *critical habitat*. Individuals found guilty of a "take" under ESA are subject to fines and/or prison.

The Sustainable Fisheries Act of 1996 reauthorized the Magnusen-Stevens Fisheries Conservation and Management Act. The Act requires regional management councils to identify essential fish habitat (EFH) for the species in the exclusive economic zone (EEZ) and to develop conservation and enhancement measures to protect that EFH.

Shellfish growers conduct a variety of activities in the day to day operations of their farms, with the potential to adversely impact salmon or bottom fish *critical or essential habitat*. Proactively the Pacific Coast Shellfish Growers Association is developing an environmental code of practice, pursuing research to better understand the interactions with protected fish species and discussing with federal and state resource management agencies the best approach to achieve some level of regulatory stability under these federal acts.

RESOURCE MANAGEMENT ISSUES FACING SHELLFISH AQUACULTURE ON THE MID-ATLANTIC COAST. Mark W. Luckenbach, Virginia Institute of Marine Science, College of William and Mary, Wachapreague, VA 23480.

Bivalve aquaculture, particularly that of hard clams *Mercenaria mercenaria*, has expanded rapidly along the mid- and south-Atlantic coasts of the United States over the past several decades. Though generally perceived as an "environmentally-friendly" and sustainable use of near-shore coastal waters, neither the ecological interactions nor the resource management implications of this use of near shore habitats have received much consideration in this region. In this presentation, I (1) briefly describe the techniques used in shellfish aquaculture in this region and (2) provide an overview of some of the research and management issues relevant to its further development and expansion throughout the region. Emphasis is placed on the need for research to understand ecological interactions with aquaculture to promote its wise and sustainable development. Understanding issues such as the role of shellfish culture operations on nutrient cycling and food web dynamics, its interactions with submerged aquatic vegetation and its impacts on fish habitat are fundamental to developing sound policies to guide its development. The inevitable conflict between increasing populations in the coastal zone and a growing aquaculture industry underscore the need for proactive strategies to promote the development of environmentally-sound, culturally acceptable aquaculture practices.

THE DEPARTMENT OF COMMERCE AQUACULTURE PROGRAM—IMPLICATIONS AND OPPORTUNITIES FOR SHELLFISH CULTURE. Ed Rhodes, NOAA Fisheries, 1315 East-West Highway, Silver Spring, MD 20910.

During the last year the Department of Commerce (DOC) has made progress in advancing its aquaculture program through a new Department aquaculture policy, the development of draft legislation for aquaculture in Federal waters, and by holding a stakeholder workshop. The shellfish aquaculture industry is a key component of the domestic production of marine species, and some pieces of the Commerce program have relevancy to this industry. The Commerce Aquaculture Policy became effective in August and outlines implementation steps for the Department that will, in cooperation with other governmental and non-governmental part-

ners, create a business climate favorable for environmentally sound aquaculture development. The policy recognizes that it is the role of government to foster economic opportunities in aquaculture by providing a fair regulatory framework within which the industry can operate, and to efficiently provide other government services, including technology development, information and financial, marketing and trade assistance. The Department has developed draft legislation that, if enacted, would authorize the Secretary of Commerce to issue long term leases for aquaculture facilities in U.S. Federal waters, generally between three miles and two hundred miles from seaward State boundaries. A significant part of this legislation is the development in a timely fashion of environmental standards for aquaculture operations that would apply to lease holders in Federal waters. DOC held a stakeholder workshop in late summer to obtain guidance for its aquaculture program. Some of the results are especially relevant to the shellfish aquaculture industry. The workshop also spawned some regional focus groups that may lead to enhanced cooperation across species groups.

THE DEVELOPMENT OF AN ENVIRONMENTAL MANAGEMENT SYSTEM FOR THE BC SHELLFISH FARMING INDUSTRY. Ruth Salmon, Executive Director, BC Shellfish Growers Association.

The BC Shellfish Growers Association (BCSGA) is proceeding with the development and implementation of an Environmental Management System (EMS) for the BC shellfish farming Industry. The EMS will comprise of an Environmental Policy (EP) and Codes of Practice (COP). The EP will set out the philosophy or approach that the industry will take to address a range of environmental issues and the COP is the tool through which that philosophy will be implemented. The development of an EMS is a key initiative for the development of BC's shellfish farming industry. The goal of this project is to provide BC shellfish farmers and processors with guidance for maintaining and protecting environmental quality while improving production and processing efficiencies. The process will allow existing farmers to objectively assess their internal operations for possible solutions towards the pursuit of an ecologically sustainable farming operation. Shellfish aquaculture is a marine-based industry that is affected by other land users such as tourism, recreation, forestry, agriculture and urban development. The effects of these industries as well as the shellfish aquaculture industry's impact on them need to be examined in a comprehensive manner to create an effective EMS. In developing an EMS, consultation with the community and other users of the marine resource will be critical to enable legitimate concerns and issues to be raised and solutions proposed. Shellfish industries in other regions of Canada and other countries are also taking a proactive role in developing policies and actions to address environmental issues.

IMPACTS OF THE STEVENS TREATIES ON WESTERN WASHINGTON TRIBAL SHELLFISH CULTURE. Derrick R. Toba,* The Tulalip Tribes, Tulalip Shellfish Program, 7615 Totem Beach Road, Marysville, WA 98271.

Shellfish and fish have been an important resource to Western Washington Indian tribes for thousands of years. This was reflected in the reservation of fishing rights in the Stevens Treaties signed in 1854 & 1855. In 1994, the federal district court ruled that the Western Washington Treaty Tribes reserved the right to harvest up to 50% of the sustainable harvest biomass of shellfish in their usual and accustomed fishing areas. However, the treaty also contained a proviso, which excluded lands that were "staked and cultivated". Following the canons of treaty interpretation, Judge Rafeedie ruled that "staked and cultivated" followed the definitions used by the shellfish industry at the time of the Treaty and by what the Indians signing the Treaty would have understood. Judge Rafeedie ruled that the shellfish industry could not "stake and cultivate" naturally occurring shellfish beds. In addition, the State of Washington sold tidelands to private individuals, which may or may not be included in the proviso.

Certiarari was denied by the U.S. Supreme Court regarding the shellfish case. However, the 9th Circuit Court of Appeals remanded several issues back to the lower court for clarification, several of which impact shellfish aquaculture. The current status of the case will be discussed.

In addition, the State of Washington, Treaty Tribes, and the Federal Government signed a shellfish sanitation consent decree in 1994, which allowed for increasing tribal responsibilities in becoming a Shellfish Control Authority. Until Tribes have that full capability, the tribes will work in conjunction with the State of Washington, which applies federal regulations regarding shellfish sanitation.

NUISANCE SPECIES

STATUS OF THE EUROPEAN GREEN CRAB INVASION IN WASHINGTON COASTAL ESTUARIES: CAN EXPANSION BE PREVENTED? Elizabeth M. Carr* and **Brett R. Dumbauld,** Washington State Department of Fish and Wildlife, P.O. Box 190, Ocean Park, WA 98640.

The European green crab *Carcinus maenas* was first found in the San Francisco estuary on the West coast of North America in 1989 and has since spread as far north as British Columbia. What is believed to be the result of a strong coast-wide recruitment event in 1997 resulted in the discovery of this invader in Washington coastal estuaries in 1998. Washington state has since responded by establishing a statewide monitoring and control program for both the invader and native crabs. Results from the monitoring program in the coastal estuaries of Willapa Bay and Grays Harbor suggest

that green crab have either declined in abundance or spread out, since average catch per unit effort (CPUE) from a location near the mouth of Willapa Bay declined from 0.008–1.04 crab/trap/hr in the summer of 1998 to 0.002–0.006 crab/trap/hr in 1999. Crabs have grown in size and ovigerous females were noted during the winter and spring months. The presence of a new year class was also noted in late summer 1999, but abundance of this year class also appeared to be much lower than that of the previous year. In general the crab prefers low salt marsh habitat during the summer months, where it is often most abundant in cover provided by *Spartina alterniflora* (another invader in Willapa Bay) or the native *Triglochin maritimum*, but does not come to traps and moves to low intertidal and subtidal habitats during the winter. Control is expected to be difficult due to a number of factors including logistics like the vast area to be covered, but also potentially declining interest from affected parties like the shellfish growers due to the lack of observed effects on their product to date with crabs at such low density. Nonetheless, it seems imperative that an effort be made to at least reduce the population and therefore further reduce the chance and success of what have already been shown to be intermittent recruitment events. With the help of volunteers we expect to initiate a broader scale control effort with traps in 2000 and are considering several ways to make this effort more effective.

PROGRESS IMPLEMENTING A PLAN TO MONITOR FOR PRESENCE OF THE EUROPEAN GREEN CRAB (*CARCINUS MAENAS*) IN PUGET SOUND, WASHINGTON. **Anita E. Cook*** and **Sandra Hanson,** Washington State Department of Fish and Wildlife (WDFW), Point Whitney Shellfish Lab, Brinnon, WA 98320.

A significant population of the European green crab, whose first persistent presence on the U.S. west coast was recorded in 1989 in San Francisco, was first noted in Washington State in 1998 in coastal Willapa Bay and Grays Harbor. The green crab likely arrived in Washington via larval drift on ocean currents. To date no European green crab have been confirmed in Puget Sound.

A large-scale Puget Sound green crab monitoring program was established in 1999, with WDFW as the coordinating agency. The primary aim of this initial phase was thorough geographical sampling coverage of Puget Sound (including the Strait of Juan de Fuca and the San Juan Islands) to maximize the potential of detecting any green crab that might have spread to Puget Sound by larval transport or other means. This was accomplished by enlisting and training various volunteers to set crayfish traps at monitoring sites spread throughout the Puget Sound. Over 15 groups sampled more than 80 monitoring stations in 1999. Participants included non-profit volunteer organizations, shellfish growers, tribes, marine science centers, government agencies, schools, and the general public. In addition to providing information about the potential presence of green crab in Puget Sound, the trapping supplied some general baseline data about populations of small native crab in the sampling areas. In the year 2000 WDFW will focus on increasing the number of sample sites (for higher potential of discovering green crab presence), identifying sites with the highest likelihood for introductions, and examining other green crab detection techniques.

BIOLOGICAL AND ECOLOGICAL ASSESSMENTS OF *NUTTALLIA OBSCURATA* IN NORTH PUGET SOUND. **Paul A. Dinnel,** Shannon Point Marine Center, 1900 Shannon Point Road, Anacortes, WA, 98221; **Erika Yates,** University of North Carolina at Pembroke, NC.

The purple varnish, or mahogany clam, *Nuttallia obscurata*, is a recent arrival to northern regions of Puget Sound. This clam is native to Japan, Korea, and China, and was probably introduced to the Pacific Northwest in the late 1980's via ballast water discharged in the Vancouver, BC region. This species has spread rapidly and may now be found as far south as Port Townsend, WA. Lack of data for this species drove this study to assess its biological and ecological characteristics in the North Puget Sound region of Washington State. During this study, we measured length/weight/width characteristics, size-frequency distribution, depth in sediment, timing of post-larval recruitment, survival and growth of the 1999 year class, and *Nuttallia's* relationship to interstitial salinity. We also assessed edibility and shelf-life, as this clam may possibly be a future sport or fishery resource. During an initial survey of ten beaches in the Padilla Bay region of North Puget Sound, we found *Nuttallia* at only three locations: one each in Padilla Bay, Fidalgo and Samish Bays at tidal elevations between +0.6 to +1.5 m (MLLW). The habitat preference of this species was clean sand or mixed sand/gravel. Most post-larval recruitment appeared to take place in late winter or early spring, with growth from 4 mm shell width in mid-June 1999 to about 11 mm by mid-October. Adult sizes ranged up to about 70 mm shell width. Densities of first-year clams in Samish Bay were about 250 clams/m^2 in mid-June and 110/m^2 in mid-October. Densities of adult clams have been found to be as high as about 800 clams/m^2 in a localized area in Fidalgo Bay. Edibility was judged to be excellent for clams less than about 35 mm shell width. Edibility of larger clams was compromised by a very "creamy texture," which was probably due to gonad development. Initial tests to assess shelf life indicated that *Nuttallia* could survive at least 30 days in a refrigerator at about 4 °C.

BIOLOGICAL INVASIONS IN COASTAL WATERS. **Andrew N. Cohen,** San Francisco Estuary Institute, 1325 South 46th Street, Richmond, CA 94804.

Over the past 20 years, a rapidly accumulating body of knowledge has demonstrated that invasions by exotic organisms threaten the aquatic flora and fauna in the world's coastal regions and the

human activities and economies that depend on them. Various invasions have disrupted food webs, altered the physical structure of ecosystems, decimated fisheries, damaged water supply systems, and driven aquaculture operations into bankruptcy. The extent of these invasions has been studied most intensively in the San Francisco Bay/Delta Estuary, which hosts over 230 exotic species including protists, plants and animals. Exotic species dominate several habitats in this estuary, accounting for 40% to 100% of the common species and over 90% of the biomass in some habitats. Furthermore, the rate of invasion has been increasing, from an average rate of about one new species a year before 1960, to nearly four new species a year since 1960. Pathways for the introduction of exotic aquatic species include ships' ballast water and hull fouling, aquaculture activities, the aquarium and ornamental plant trades, and the live bait and seafood trades. With the continuing expansion of international trade there will be an ever-increasing risk of introduction of exotic organisms—including parasites and diseases of fish and shellfish and human parasites and diseases that may be transferred through the consumption of fish and shellfish—unless stronger measures are adopted to manage these invasion pathways.

PRO-ACTIVE MANAGEMENT OF INTRODUCED MARINE PESTS: LESSONS FROM THE APPARENTLY SUCCESSFUL ERADICATION OF THE SABELLID WORM IN CALIFORNIA. Carolynn S. Culver* and **Armand M. Kuris,** Marine Science Institute and Ecology, Evolution and Marine Biology, University of California, Santa Barbara, CA 93106.

Although much effort has recently been devoted to prevention of additional introductions of non-indigenous species, little, if anything, has been done to eradicate or control those pests that are already here. This lack of a pro-active stance towards established invaders is, in part, due to the perception that once an invader has become established, nothing can be done to reduce its associated impacts. In addition, others take a "wait & see" attitude, where substantial negative impacts must be shown before even considering development or implementation of eradication/control measures. Some recent experiences suggest that these defeatist attitudes may be unwarranted and result in costly delays that allow pest populations to increase and spread. Subsequently, the chance for successful eradication/control is decreased, while the likelihood for damage to the ecosystem and the costs associated with management of the pest are increased. To minimize such negative outcomes, a more pro-active management stance should be considered. The apparent eradication of an introduced population of the South African sabellid worm pest in California offers insight towards development and implementation of a successful, cost-effective management program. We will discuss criteria for successful management of invasive species and review the need and potential for eradication/control of other introduced marine pests.

PREDATION BY EUROPEAN GREEN CRABS ON MANILA CLAMS IN CENTRAL CALIFORNIA. Edwin Grosholz and **Paul Olin,** Department of Environmental Science and Policy, University of California, Davis, CA 95616 and University of California Sea Grant Extension Program, 2604 Ventura Avenue, Room 100, Santa Rosa, CA 95403.

One of the key concerns regarding the recent invasion of the European green crab, *Carcinus maenas,* is the potential impact of this species upon invertebrate fisheries such as clams, oysters, and mussels in western North America. To investigate the potential impacts of green crabs upon the Manila clam (*Venerupis philippinarum*) fishery, we conducted a field experiment to determine size-specific rates of predation by green crabs on Manila clams. Using commercial growout bags provided by Hog Island Oyster Company of Marshall, CA, we placed one green crab from one of three sizes classes (30–40 mm, 50–65 mm, or >70 mm) into a growout bag with fifteen Manila clams chosen from one of three size classes (<23 mm, 25–33 mm, or >36 mm), the largest size class being market size. Five replicate bags of each of the nine treatments (three crab sizes by three clam sizes) were placed near MLLW on July 13, 1999 and lightly covered with sediment. We used the five replicates of the small green crab/large Manila clam treatment as conservative controls. After two weeks, we collected all bags and assessed the mortality of clams and crabs. We found very low survival of Manila clams in treatments with large green crabs. For the smallest Manila clams in treatments with large crabs, nearly all clams were eaten with survivors remaining in only one bag (15% overall). Small clams had moderately better survival in treatments with medium (52%) and small green crabs (71%). The medium size class of clams had poor survival in bags with either large (36%) or medium sized green crabs (46%). The large market size clams had 65% survival with both medium and large green crabs over the two week period. In summary, our results show that even newly recruiting juvenile green crabs can quickly reduce the numbers of juvenile Manila clams, and even market size clams are at risk from even intermediate size green crabs. Therefore we conclude that green crab predation may represent a significant threat to the commercial production of Manila clams.

THE IMPACT OF EUROPEAN GREEN CRABS IN CENTRAL CALIFORNIA. Edwin Grosholz, Department of Environmental Science and Policy, University of California, Davis, CA 95616; **Gregory Ruiz,** Smithsonian Environmental Research Center, P.O. Box 28, Edgewater, MD 21037.

The European green crab, *Carcinus maenas,* is one of the most potentially serious recent introductions into the coastal waters of western North America. In this study, we measured the impacts of green crabs on a coastal marine food web in central California. We found that this predator exerted strong "top-down" control and significantly reduced the abundances of several of the 20 invertebrate species monitored over a nine-year period. Densities of na-

tive clams, *Nutricola tantilla* and *Nutricola confusa*, and native shore crabs, *Hemigrapsus oregonensis*, declined fivefold to tenfold within three years of the green crab invasion. Field and laboratory experiments indicated predation by green crabs caused these declines. In addition, we tested for indirect responses of invertebrates and vertebrates to green crab predation. We found significant increases in the abundances of two polychaete taxa, *Lumbrineris* sp. and *Exogene* sp., and tube-building tunaid crustaceans, *Leptochelia dubia*, most likely due to the removal of co-occurring green crab prey. However, we observed no significant changes in shorebird abundances (13 species) over a nine-year period suggesting green crabs have had no "bottom-up" effect on shorebird populations, which subsist on benthic invertebrate prey. We predict that such "bottom-up" control may occur as both the local effects and the geographic range of green crabs increase.

POTENTIAL LIMITATIONS OF THE EUROPEAN GREEN CRAB, *CARCINUS MAENAS*, IN HABITAT SUITABLE FOR THE NATIVE RED ROCK CRAB, *CANCER PRODUCTUS*. **Chris Hunt,*** Environmental Science Department, Oregon State University, Corvallis, OR 97331-2914.

Carcinus maenas, thought to have arrived in the Western Pacific in San Francisco Bay in 1989, was discovered in Coos Bay, Oregon in 1997. By the summer of 1998 *C. maenas* was discovered in at least eight Oregon estuaries. Trapping observations during the summer of 1998 suggested that low *C. maenas* abundance occurred in areas that were either physiologically intolerant for *C. maenas*, or areas occupied by large numbers of adult *Cancer productus*, the native Red Rock crab. An intensified trapping effort in Yaquina Bay, Oregon, during the summer of 1999 was used to document the distribution of the estuary's crab community. These observations indicated that although *C. maenas* coexisted with adult Dungeness crab, *Cancer magister*, it was much more rare in areas where the physiologically more sensitive adult *C. productus* were abundant. These results are further supported by lab predation studies pairing adult and juvenile crabs of both *C. maenas* and *C. productus*. These two observational studies support the theory that in habitat suitable for adult *C. productus*, the invasive *C. maenas* may be severely restricted. This research was supported by Oregon Sea Grant.

EAST MEETS WEST: COMPETITIVE INTERACTIONS BETWEEN GREEN CRAB AND *HEMIGRAPSUS* SPP. **Gregory C. Jensen,* P. Sean McDonald,** and **David A. Armstrong,** School of Fisheries 355020, University of Washington, Seattle, WA 98195.

Juvenile green crab (*Carcinus maenas*) rely on intertidal structure (i.e., rocks, shell) for shelter, and the recent introduction of this species to the west coast of North America places them in potential competition for this resource with the abundant native grapsid, *Hemigrapsus oregonensis*. Similarly, the recent arrival of a Japanese species, *H. sanguineus*, on the east coast of North America also suggests the possibility for competitive interactions. The morphological and behavioral similarities of these two grapsids and their likely interaction with juvenile *Carcinus* provides an interesting contrast, with *Carcinus* in the role of invader on the west coast and as "resident" on the east coast, having been established there for 150+ years. We conducted fine-scale sampling on both coasts, examining species distributions both under rocks and in adjacent sediments. Only 20% of the juvenile *Carcinus* sampled were found under rocks in areas occupied by either *Hemigrapsus* species, while north of the present distribution of *H. sanguineus* >97% of the *Carcinus* were under rocks. In laboratory trials examining competition for food or space between *Carcinus* and *Hemigrapsus* of equal carapace width, *H. sanguineus* was overwhelmingly dominant, and *H. oregonensis* also dominated in competition for space. These findings may have important implications both for the ultimate distribution and impact of *Carcinus*, and also for possible use of grapsids for biocontrol in culture systems.

THE POTENTIAL IMPACTS OF *CARCINUS MAENAS* INTRODUCTION ON JUVENILE DUNGENESS CRAB, *CANCER MAGISTER*, SURVIVAL. **P. Sean McDonald,* Gregory C. Jensen,** and **David A. Armstrong,** School of Fisheries, University of Washington, Seattle, WA 98195.

The spread of the European green crab, *Carcinus maenas*, in the northeast Pacific represents one of many invasive introductions that are potentially devastating to aquaculture and shellfisheries. Discovery of the species in Washington State coastal estuaries in 1998 precipitated debate as to the impact of the invasion on local commercial and recreational Dungeness crab, *Cancer magister*, harvests. These estuaries provide appropriate habitat for *C. maenas*, yet they are important nursery grounds for *C. magister*. The results of laboratory experiments and infrared video observations show that juvenile *C. maenas* displace *C. magister* of equal size from single shells in one-on-one competition. *C. maenas* also consistently wins nocturnal foraging trials in which the species compete for freshly killed clams. Laboratory and field enclosure experiments indicate that juvenile *C. magister* emigrate from refuge habitat as a result of competition and predation by adult *C. maenas*. Interactions with the dominant invasive species could have a negative influence on juvenile *C. magister* survival and subsequent recruitment to the fishery. However, the real impact of the *C. maenas* introduction will depend on the extent to which the two species actually overlap, a condition which may be more limited than previously thought.

THE STATUS OF AQUATIC NUISANCE SPECIES PREVENTION IN WASHINGTON STATE. Pam Meacham, Washington Department of Fish and Wildlife, 600 Capitol Way N., Olympia, WA 98501.

Aquatic nuisance species pose a threat to the ecological integrity of Washington's marine and freshwater resources, and have a significant impact on economic, social, and public health conditions in Washington State. We have learned from our experience with spartina that these species can spread rapidly, and we must do everything in our power to prevent new introductions and minimize the impact of those already present.

The state hired a full time ANS Coordinator, established a Zebra Mussel and Green Crab Task Force, and provided funding for ANS programs. A State ANS Management Plan has been completed, and monitoring and control plans for green crab and zebra mussels have been put in place. Presently there is a multi-agency focus on educating the public on the role they can play in preventing the spread of ANS plants and animals. Two pieces of legislation have been drafted for the 2000 legislature. One bill, supported by the shipping industry, is designed to protect Washington waters from the introduction of non-native organisms and pathogens carried in ballast water and other ship vectors. The other bill creates an Aquatic Nuisance Coordinating Committee with the intent of minimizing the environmental and economic risks of ANS by enhancing cooperation and coordination among the various state and federal agencies responsible for controlling ANS. Through the cooperative efforts of federal, state, and local government, industry, and the public, Washington State is making a significant contribution toward solving a global problem.

THE EUROPEAN GREEN CRAB BIVALVE CONSUMPTION RATES AND PREY PREFERENCES. Kelly C. Palacios,* College of Oceanic and Atmospheric Sciences, 104 Ocean Admin. Bldg., Corvallis, OR 97331-5503; **Steven P. Ferraro,** Coastal Ecology Branch, US EPA, Newport, OR 97365.

The European green crab, *Carcinus maenas*, a voracious bivalve predator, is a recent invader to Pacific Northwest estuaries. The objectives of this study were to determine green crab consumption rates and prey preferences using four bivalve species: Yaquina oyster (*Ostrea lurida*), Manila clam (*Tapes phillinarum*), bent-nosed clam (*Macoma nasuta*), and Cryptomya clam (*Cryptomya californica*). Various bivalve size classes, ranging from the smallest (10–14 mm) to the largest (33–37 mm), were tested. In both the consumption and preference experiments ($\eta = 3 - 8$), one previously starved (48 hours) green crab (CW: 60–75 mm) was placed in a 38 l aquaria with 13 cm of sediment and allowed to feed ad libitum on bivalve prey for 16 hours. For each bivalve species being tested, 60 individuals were offered at the beginning of the experiment and not replaced. Differences in the mean consumption rates were tested by ANOVA. The null hypothesis of no prey preference was tested by a goodness-of-fit (G-test) to an equal proportion of prey consumed. Among the prey species tested, there was no difference in the mean consumption rate for a given prey size class, but green crabs exhibited strong prey preferences when offered more than one prey species choice. The results suggest that Yaquina oysters are at greater risk of green crab predation than bent-nosed clams and Manila clams and Cryptomya clams are at greater risk than bent-nosed clams.

ABUNDANCE OF SMALL PREDATORY GASTROPODS (*UROSALPINX CINERA*, *EUPLEURA CAUDATA*, *RAPANA VENOSA*) IN RELATION TO LOWER CHESAPEAKE BAY OYSTER (*CRASSOSTREA VIRGINICA*) POPULATIONS. Melissa J. Southworth,* Juliana M. Harding, and **Roger Mann,** Department of Fisheries Science, Virginia Institute of Marine Science, Gloucester Point, VA 23062.

Oysters in the Virginia portion of the Chesapeake Bay have enjoyed a relative hiatus from oyster drill (*Urosalpinx cinera*, *Eupleura caudata*) predation since Hurricane Agnes dramatically reduced oyster drill abundance in 1972. In recent years, anecdotal reports have indicated that oyster drill abundance has been increasing in Virginia waters. Increased oyster drill abundance combined with the recent discovery of a third predatory gastropod, Veined Rapa whelk (*Rapana venosa*), in the lower Chesapeake Bay, has potentially significant consequences for the commercial oyster fishery as well as Virginia's ongoing oyster restoration efforts. Quantitative estimates of the abundance and distribution of small predatory gastropods in relation to existing oyster resources were made during Fall 1999 at >150 sites in 8 tributaries. Oyster drills were present in <50% of sites sampled, juvenile *Rapana venosa* were not observed. Both species of oyster drill were more abundant in downriver habitats with salinities ranging from 15 to 25 ppt. Drill abundance ranged from 1 to 4 animals m^{-2}.

STATUS OF THE CHINESE MITTEN CRAB IN CALIFORNIA. Tanya C. Veldhuizon,* California Department of Water Resources, Environmental Services Office, Sacramento, CA 95816.

The catadromous Chinese mitten crab (*Eriocheir sinensis*) is native to China and Korea and is also established in Europe and California. First collected in south San Francisco Bay in 1992, *E. sinensis* rapidly expanded in distribution and abundance. The current distribution in California is the San Francisco Estuary and the lower elevational reaches of the watershed. Based on the adverse impacts of the crab in Germany, *E. sinensis* poses ecological, economic, and health concerns in California. However, an assessment of the degree of impact in California is required. In 1999, the California Fish and Game Commission denied requests to commercially exploit the crab. Reasons for denial ranged from potential acceleration of dispersal to increased management costs to encouragement of future illegal introductions. Research and man-

agement of *E. sinensis* in California are facilitated through the Interagency Ecological Program's (IEP) Chinese mitten crab Project Work Team. For additional information, visit the IEP website at <http://www.iep.ca.gov>.

MITIGATING EFFECTS OF NONINDIGENOUS MARINE SPECIES: EVALUATION OF SELECTIVE HARVEST OF THE EUROPEAN GREEN CRAB, *CARCINUS MAENAS*. **William C. Walton,*** Smithsonian Environmental Research Center, PO Box 28, Edgewater, MD 21037.

With the increasing need for management of nonindigenous species in marine habitats, managers are considering available mitigation methods to reduce the negative effects of established exotic species. I briefly outline a menu of possible mitigation methods, illustrated by management experience with the European green crab, *Carcinus maenas:* chemical control, biological control, genetic manipulations, local physical barriers, altered maritime/fishery practices, and selective harvest (trapping). Selective harvest, relative to the alternatives is generally perceived as incurring the least negative side effects on resident species. Does selective harvest, however, sufficiently reduce invader abundance to significantly reduce their negative effects? As a case study, I explore the efficacy of municipal selective harvest programs currently in use on Martha's Vineyard, MA (USA). Current harvest programs, despite considerable effort, do not appear to reduce the within- or among-year abundance of *C. maenas* (verified by independent censuses). Experimental tests of intensive, short-term trapping in the shallow subtidal zone (every 48 hrs for 2 weeks) similarly led to no decline. Habitat-specific trapping surveys and mark-recapture studies, however, suggest that populations within embayments are relatively closed and therefore theoretically vulnerable to *within-year* reductions in abundance given appropriate harvest levels. I tested this with an intensive one day trap down (6 hauls, 1.5 hr immersion time) in a relatively small embayment (~0.01 km^2) and observed significant declines in 1) green crab abundance and 2) relative predation intensity on quahaug, *Mercenaria mercenaria*, seed (13–17 mm shell length). The reduction in relative predation intensity persisted for up to a month. Selective harvest as currently practiced, therefore, does not appear to be effective, but deserves further consideration as a possible mitigation method.

INTEGRATING BIOLOGICAL CONTROL IN THE INTEGRATED PEST MANAGEMENT PROGRAM FOR SPARTINA ALTERNIFLORA IN WILLAPA BAY. **Miranda Wecker,** Marine Program, Olympic Natural Resources Center, University of Washington; **Donald Strong,** Center for Population Biology; **Fritzi Grevstad,** Olympic Natural Resources Center, University of Washington.

In 1995, the Washington Legislature unanimously declared the spread of invasive exotic Spartina "an environmental disaster." (RCW 17.26.005). Extensive research supported the conclusion that Spartina species are causing profound structural and, if not controlled, irreversible alterations to estuarine areas. Since 1993, over $1,000,000 has been spent each biennium by state and federal agencies on an "integrated pest management" (IPM) approach to Spartina control. Still the pace of spread outstrips the rate of control. Ecologically sound, effective, and affordable new control techniques are needed to achieve the program's goals. Biological control is considered the most promising new tool for Spartina control in Willapa Bay, the site of the largest infestation. Greenhouse studies carried out in the early 1990s demonstrated that Willapa Spartina clones were severely stressed or killed by moderate populations of Prokelisia marginata, a leafhopper common to Spartina's home range (Daehler & Strong 1997).

Research was undertaken during the past two years to evaluate the risks of releasing P. marginata in Washington state. Host specificity studies included choice, no-choice and preference trials during which the most likely non-target hosts were exposed to *P. marginata*. Tests were also conducted to determine whether *P. marginata* serves as a vector for pathogens responsible for observed mortality of Willapa Spartina. Preparations are underway for release of the insects. Pre-release monitoring has begun and a release strategy is being formulated. Project participants are also testing new approaches to the transfer of scientific information generated through research activities. The team will prepare sophisticated models that will allow participants to project and track the spread and impacts of the insects. Using these planning tools, state agency officials will be able to comprehensively target the use of other control techniques and generate a more efficient overall plan. Extensive outreach activities have accompanied the scientific studies in order to promote public understanding of the project and a sophisticated appreciation of its findings. A project website will permit the public and professional managers access to accumulated information, project status reports and analytic products.

GROWTH OF THE 1997/1998 YEAR CLASS OF THE EUROPEAN GREEN CRAB, *CARCINUS MAENAS*, IN OREGON ESTUARIES. **Sylvia Behrens Yamada, Chris Hunt,*** and **Alex Kalin,** Zoology Department, Oregon State University, Corvallis, OR 97331-2914.

During the summer of 1998, a new year class of *Carcinus maenas* appeared in Oregon estuaries as well as in Humboldt Bay, CA to the south and Willapa Bay, WA, Grays Harbor, WA and Vancouver Island, B.C. to the north. This coast-wide colonization is correlated with an El Niño event of unusually strong northward moving coastal currents from September 1997 to spring of 1998. Crabs from the 1997/98 year class grew quickly, averaging 14 mm in carapace width in June, 27 mm in July and 47 mm in September 1998. Growth ceased during the winter, resumed in May 1999 and by the end of their second summer, they averaged 70 mm.

Female *Carcinus maenas* from the 1997/98 year class carried eggs in November and December 1998. Sexual maturity in Oregon populations is thus reached within one year, while in the North Sea and Maine it may take two to three years. A comparison of molt increments of crabs from Oregon, the North Sea and Maine indicates that growth per molt is constant regardless of geographic location. *Carcinus maenas* in Oregon therefore molt more frequently than in the North Sea or in Maine. The trade-off for this faster growth rate is a shorter life span. While the life span for *C. maenas* is 5–6 years in Maine, it may only be around 3 years in Oregon.

While *C. maenas* reproduced in Oregon estuaries during the winter of 1998/1999, the resulting offspring is not replacing the parental year class. Recruitment during the summer of 1999 was late and sparse. If recruitment in the next few years is also low, then the resident *C. maenas* populations in Oregon estuaries will die out until another coast-wide colonization event occurs with the next El Niño. This research was supported by Oregon Sea Grant.

PHYTOPLANKTON HARMFUL TO SHELLFISH AND CONSUMERS

DOES THE HISTORY OF TOXIN EXPOSURE INFLUENCE BIVALVE POPULATION RESPONSES TO PSP TOXINS IN *MYA ARENARIA*?: I) BURROWING AND NERVE RESPONSES. **V. Monica Bricelj,*** Institute for Marine Biosciences, National Research Council (NRC), 1411 Oxford St., Halifax, NS B3H 3Z1, Canada; **Betty M. Twarog,** Darling Marine Center, Univ. of Maine, Walpole, ME 04573, USA; **Scott P. MacQuarrie** and **Pamela Chang,** NRC, Halifax, and **Vera L. Trainer,** Northwest Fisheries Science Center, Seattle, WA 98112-2097, USA.

Our ECOHAB (National Program on the Ecology and Oceanography of Harmful Algal Blooms) study examines the magnitude and causes of intraspecific variation in sensitivity to paralytic shellfish poisoning (PSP) neurotoxins, and thus capacity for toxin accumulation, in North American populations of softshell clams, *Mya arenaria*. Our results suggest that *Mya* populations recurrently affected by toxic blooms may experience genetic or epigenetic adaptation to PSP toxins via natural selection of more resistant individuals. Individual sensitivity is here measured by inhibition of clam burrowing response after laboratory exposure to a highly toxic dinoflagellate, *Alexandrium tamarense* (strain PR18b), and *in vitro* block of the action potential in isolated nerves exposed to saxitoxin (STX). Burrowing inhibition was not induced by a non-toxic strain of *A. tamarense*. The percentage of sensitive clams (as determined by the burrowing index after 24 hrs. of toxin exposure) within a juvenile population ranges from 72–96% in areas with no toxin history (e.g., Lawrencetown River Estuary, LE, Nova Scotia, and Mount Sinai Harbor, Long Island, New York), to ≤15% in areas with a long-term history of toxic blooms (Lepreau Basin, LB, Bay of Fundy, New Brunswick). Differences of more than an order of magnitude in nerve sensitivity to STX were observed among individuals from LB and LE populations. Most LE clams exhibited marked reduction of the nerve action potential at 10^{-6} g STX/ml, and were fully blocked within 20 sec at 10^{-5}. In contrast, most LB clams displayed no effect even at 10^{-5} and required 3–5 min. of exposure to induce full nerve block at 10^{-4} g STX/ml. The effects of duration of toxification and depuration were also tested: neither variable affected the nerve sensitivity of LB clams. There was an apparent decrease in the sensitivity of LE clams which survived prolonged (15-day) toxification, compared to those exposed for 4–6 days, but this effect might be attributed to selective mortality of the most sensitive LE phenotypes over time. Relative merits of the toxin sensitivity indices used are discussed. We will attempt to identify adaptive mechanisms to toxins at the biochemical and molecular level, by determining the presence of soluble toxin-binding saxiphilins in various clam tissues, or genes encoding for these proteins, as well as by DNA sequencing of sodium channel STX receptor sites.

HARMFUL ALGAL BLOOMS AND SHELLFISH TOXICITY IN WASHINGTON STATE. **Rita A. Horner,*** School of Oceanography, Box 357940, University of Washington, Seattle, WA 98195-7940; **Frank H. Cox** and **Linda D. Hanson,** Washington Department of Health/Shellfish Programs, P.O. Box 47824, Olympia, WA 98504-7824.

Harmful algal blooms (HABs) and the toxins they produce are an increasing threat to human health and fisheries resources around the world. In western Washington marine waters, fewer than 20 phytoplankton species may produce marine toxins and are frequent members of the phytoplankton community. Both the phytoplankton species and the toxins they produce are most common from April through October, but occur in all months of the year. Blooms last a few days or several months, while the toxins in shellfish usually last for weeks to months. Potentially harmful species may be present, but produce little or no toxin or, conversely, only a few cells may produce high levels of toxin. Environmental factors that control the presence of harmful species and toxin production are not well-known here and vary with the algal species, locality, season, and year; population dynamics are poorly understood. The current management tool is to close a fishery or area if toxins are present in a product which means that broad geographic areas and all shellfish species are involved when possibly only a small area or a few species are affected.

IMPACT OF HARMFUL DINOFLAGELLATE *HETEROCAPSA CIRCULARISQUAMA* ON SHELLFISH AQUACULTURE IN JAPAN. Yukihiko Matsuyama* and **Takuji Uchida,** National Research Institute of Fisheries and Environment of Inland Sea, Ohno, Hiroshima 739-0452, Japan; **Tsuneo Honjo,** Faculty of Agriculture, Kyushu University, Hakozaki, Fukuoka 812-8581, Japan.

The novel dinoflagellate *Heterocapsa circularisquama* Horiguchi has been the causal agent of red tide on the Japanese coast since 1988. The red tide due to *H. circularisquama* has destroyed the shellfish aquaculture industries around the western part of Japan. Until 1998, 26 cases of *H. circularisquama* red tide (including 15 incidences leading to fisheries damage) had been recorded in 14 locations of western Japan. The red tide due to *H. circularisquama* was associated with massive killing of commercially important bivalve species: short-necked clam *R philippinarum*, Pacific oyster *Crassostrea gigas*, pearl oyster *Pinctada fucata*, blue mussel *Mytilus galloprovincialis edulis*, etc. Economic losses of shellfish aquaculture by direct killing of marketable products were estimated about at least 10 billion-yen in the last decade The laboratory experiments demonstrated that *H. circularisquama* reduces the clearance rate of bivalves at the density of 2–104 cells/l, and kills them at 5–106 cells/l in association with vigorous "clapping", retraction of mantles and gills, valve closure, and alternation of cardiac activities. Although the toxicity of *H. circularisquama* to bivalves and gastropods is extraordinary, any fish killing, toxin accumulation, and subsequent human illness have not been observed during blooms of this species.

METHODS FOR DETECTING MARINE TOXINS. James Hungerford,* Ronald M. Manger, Sue Lee, Linda Leja, Charles Kaysner, and **Marleen Wekell,** Seafood Products Research Center, Pacific Regional Laboratory Northwest, USFDA, Bothell, WA.

Detection of marine toxins is a crucial aspect of seafood safety. Animal bioassays have for years been the mainstay of many monitoring programs. Alternative detection methods are now being emphasized, as replacement of animal bioassays is a goal for many health agencies. Maintaining the present level of protection with new detection methods and without detailed risk assessment data implies the need to duplicate or at least parallel the observed animal assay response. This complicates methods development, since most marine toxins occur in several different forms. Toxin multiplicity is observed in animal assays as a "response profile" averaged over the toxin profile. Duplicating the response profiles of animal bioassays is challenging. Chromatographic methods can only accomplish this by separating and detecting all toxins contributing to total potency. This often requires expensive and scarce multiple toxin standards. Immunoassays would seem ideal candidates, and yet the need to conjugate hapten-scale toxins when raising the antibodies can and often does change the desired response profile. Most marine toxins are neurotoxins and many are sodium channel active. For this reason there has been considerable effort to develop assays for sodium channel toxins such as paralytic shellfish toxins, brevetoxins, and others by their toxic effects or by sodium channel binding. These include direct detection of membrane electrochemical effects, competitive binding assays using radiolabeled toxins with synaptosomes, and cytotoxicity assays using cultured nerve cells. In our laboratory a cytotoxicity assay was developed using a colored indicator of cell viability (mitochondrial dehydrogenase activity). This assay has been used to detect both sodium channel blockers such as the paralytic shellfish toxins and also sodium channel enhancers like the brevetoxins and ciguatoxins. We have transferred this technology to several laboratories and will soon run a small-scale validation study.

DOES THE HISTORY OF TOXIN EXPOSURE INFLUENCE BIVALVE POPULATION RESPONSES TO PSP TOXINS IN *MYA ARENARIA*?: II) FEEDING, SURVIVAL AND TOXIN ACCUMULATION. Scott P. MacQuarrie* and **V. Monica Bricelj,** National Research Council of Canada, Institute for Marine Biosciences, Halifax, Nova Scotia, Canada, B3H 3Z1.

The Bay of Fundy, Eastern Canada and the Gulf of Maine, USA, experience annual, recurrent paralytic shellfish poisoning (PSP) outbreaks, which negatively impact the extensive softshell clam, *Mya arenaria*, fisheries in these regions. Two *M. arenaria* populations, one with a history of recurrent, annual toxin events (Lepreau Basin, LB, New Brunswick) and one with no history of toxin exposure (Lawrencetown River Estuary, LE, Nova Scotia), were compared in their responses to PSP toxins during laboratory exposure to *Alexandrium tamarense* (strain PR18b, ca. 60 pg. saxitoxin equivalents $cell^{-1}$). Repeated measurements of the same individuals showed that significant differences in feeding rates and % burrowing between the two populations were maintained throughout the experimental period, indicating that these responses do not acclimate with prolonged (two-week) toxin exposure. Clearance rates were 4–8 times higher in *M. arenaria* from Lepreau Basin than in *M. arenaria* from Lawrencetown, and 54 to 88% of the LB clams were resistant (capable of burrowing) whereas 86 to 98% of the LE clams were sensitive (unable to burrow). Lawrencetown *M. arenaria* (non-burrowers) reached mean peak toxicities of 5000 μg STXeq 100 g^{-1} visceral mass after 24 hrs of exposure and remained at that level. The Lepreau Basin population (burrowers), however, continued to accumulate toxins but in a cyclic or fluctuating pattern, reaching toxicity levels up to 10× those of the Lawrencetown population at 7 and 15 days of toxification. Thus population differences in feeding and burrowing during toxin exposure are reflected in their differential ability to accumulate toxins. Most importantly, LB clams exhibited >98% survival while LE clams suffered cumulative mortalities of

32%, which started after one week of toxin exposure. A second experiment was undertaken in which enrichment of rare phenotypes (LB sensitive and LE resistant clams) allowed more detailed investigation of inter- and especially intrapopulation variation. Percent mortality varied greatly among the four groups, ranking as follows: LE sensitive > LE resistant >> LB sensitive > LB resistant. In this presentation feeding and toxin uptake rates from this experiment will be discussed. This study demonstrates that blooms of PSP-producing dinoflagellates can cause both lethal and sublethal effects on *Mya arenaria*, but that these effects vary in their expression both within and among populations.

DOMOIC ACID TOXICITY: PRACTICAL SOLUTIONS FOR ORGANIZATIONS TO REDUCE THE IMPACT. John S. Ramsdell, Marine Biotoxins Program, NOAA-National Ocean Service, Charleston, SC 29412.

Domoic acid is a tricarboxylic acid produced by certain species of the diatom genus *Pseud-nitzchia*. It was identified as the causative agent of the amnesic shellfish poisoning in 1987 and since that time the toxic algae has been determined in many regions of the world. Substantial toxicological data have been generated since 1987 for domoic acid effects on mammals. This presentation will summarize the major points about domoic acid toxicity, including its toxicokinetics, adverse effects and mechanisms of susceptibility. This information will then be used to discuss practical approaches that can reduce the impact of domoic acid toxicity. These approaches will include accurate communication of the hazards, identification of high risk groups, and the prospects for biomonitoring.

APPLICATION OF DNA PROBES FOR DETECTION OF HARMFUL ALGAE. Chris A. Scholin,* Monterey Bay Aquarium Research Institute, 7700 Sandholdt Rd., Moss Landing, CA 95039.

Common problems associated with monitoring waters for harmful algal bloom (HAB) species are distinguishing between potentially toxic and non-toxic organisms, and quantifying the potentially toxic species in discrete water samples routinely at many locations. Toxin-producing diatoms of the genus *Pseudo-nitzschia* are one group of organisms that exemplify these difficulties. Toxic species are those that produce domoic acid (DA), the causative agent of amnesic shellfish poisoning (ASP). At the genus level, toxic and non-toxic species of *Pseudo-nitzschia* are readily identifiable, but discriminating between different species can be time consuming due to a need for detailed morphological analysis. Species-specific DNA probes are now available for a number of *Pseudo-nitzschia* species. These probes have been evaluated in a variety of locations around the US and elsewhere in the world. In New Zealand, the probes are used routinely in commercial shellfish growing areas as part of a DA risk assessment strategy. This presentation will focus on the use of the probes as research tools, the process by which they are being evaluated in field studies, and their successful integration into monitoring programs like that in New Zealand. Defining needs of the end-users of the probes will be emphasized. Efforts to develop novel instrumentation for *in situ*, autonomous detection of HAB species will be summarized.

HARMFUL ALGAL BLOOMS AND SHELLFISH AQUACULTURE: IMPLICATIONS FOR THE FUTURE OF THE INDUSTRY. Sandra E. Shumway,* Natural Science Division, Southampton College of Long Island University, Southampton, NY 11968.

Scientists and resource managers now generally agree that the number and frequency of harmful algal blooms (HABS) are increasing over time. Many blame (sometimes inaccurately) HAB outbreaks for the loss of shellfish growing areas and impacts on aquaculture operations. HABs occur throughout the world and, in some regions, are commonplace and seasonal, while in other areas, rare or unusual. HABs can have far-reaching effects on coastal ecosystems, including ecosystem integrity, species interactions, and aquatic animal health. They can also create significant impacts on population growth, human health, local and regional economies, industry, and business. For many obvious reasons, algal species associated with HABs that affect human health continue to receive the most attention, with commercially important fish and filter feeding shellfish being the primary organisms of concern. However, these algal species are not the only ones of importance when it comes to animal health, ecosystem condition, or socioeconomic factors, and many other fish species can also be impacted. In many cases, the societal response to these outbreaks focus on mitigation and control of these adverse effects. This presentation will review current knowledge of HAB-shellfish interactions worldwide and suggest ways in which shellfish aquaculture may be undertaken successfully in the face of potential HAB outbreaks. The important interactions between science and management will be emphasized, as well as ways in which shellfish aquaculture ventures may operate without imposing undue ecological stress and operational expense.

BEHAVIORAL VARIABILITY OF THE TOXIC DINOFLAGELLATE, *PFIESTERIA PISCICIDA*, WHEN INTRODUCED TO LARVAL AND ADULT SHELLFISH. Jeffrey Springer,*[1] Sandra E Shumway,[2,3] and JoAnn Burkholder,[1] [1]North Carolina State University-Center for Applied Aquatic Ecology, Raleigh, NC 27695 USA, [2]Southampton College of Long Island University, Southampton, NY 11968 USA, and [3]Bigelow Laboratory for Ocean Sciences, West Boothbay Harbor, ME 04575.

The toxic estuarine dinoflagellate, *Pfiesteria piscicida*, is a causative agent of major fish kills in estuaries of the mid-Atlantic and southeastern U.S. *P. piscicida* zoospores are unique among

most toxic dinoflagellates in that toxic strains exhibit directed attack behavior towards live finfish, and produce toxin(s) which strip epidermal tissue from finfish and impair the nervous system. In this study we observed toxic zoospores kill *Argopecten irradians* and *Crassostrea virginica* pediveligers within 60 seconds of zoospore introduction, followed by active consumption of the shellfish and encystment within the visceral cavity. At 25 psu, the attack behavior was most pronounced toward larvae that had discarded their velum, whereas larvae with active, extended vela appeared to discourage zoospore attack and feeding behavior. However, at 15 psu zoospores of the same toxic isolate showed little attraction or attack behavior toward oyster pediveligers. We also tested the response of this clonal isolate to adult *C. virginica*. Zoospores were actively cleared from suspension within a 24 hour period with no evidence of narcosis as has been reported for other toxic, clonal isolates of *P. piscicida*. Examination of fecal material indicated that the zoospores had formed temporary cysts, and had not been adversely affected by their passage through the digestive tract. Within 24 hours, 90% of the zoospores had excysted and regained motility. The data indicate that *P. piscicida* zoospores can show striking variability in response to shellfish, both at the species level (in timing of response), and within a species depending on the life cycle stage and the salinity.

DOMOIC ACID PRODUCTION BY *PSEUDO-NITZSCHIA PSEUDODELICATISSIMA* OFF THE CENTRAL WASHINGTON COAST IS LINKED TO RECORD LEVELS OF TOXIN IN RAZOR CLAMS. **Vera L. Trainer,* Nicolaus G. Adams,** and **John C. Wekell,** National Marine Fisheries Service, Northwest Fisheries Science Center, 2725 Montlake Blvd. E., Seattle, WA 98112; **Mitch Lesoing,** Quileute Natural Resource, Quileute Indian Tribe, 234 Front St., LaPush, WA 98350.

In the early fall of 1998, record levels of domoic acid were measured in razor clams on the central WA coast within 18 days of a nearly monospecific bloom of *Pseudo-nitzschia pseudodelicatissima*. Field samples, consisting of 90–100% *P. pseudodelicatissima* (up to 15 million cells/L seawater) were found by mass spectroscopy to contain up to approximately 4 μg domoic acid/L seawater. Measurement of increasing levels of toxin in a cultured isolate of this species using a receptor binding assay, showed that this diatom is a domoic acid-producer in WA coastal waters. The economic impacts of this single bloom in 1998 were over $15–20 million dollars due to the coastwide loss of razor clam harvest. Because of the slow depuration of razor clams and the variability of domoic acid levels measured in these bivalves, harvest closures occurred also in the fall of 1999, again resulting in millions of dollars in losses to the already economically-stressed coastal communities. The 1998 bloom of this pennate diatom was preceded by strong coastal upwelling in early September, indicated by high levels of silicate and nitrate at a time of anomalously low rainfall, a typical phenomenon in post-El Niño years. Subsequent wind relaxation and reversal events are suggested to have resulted in the Ekman transport of surface cell populations into nearshore waters where nutrients were plentiful, providing optimal conditions for bloom development.

THE DISTRIBUTION OF DOMOIC ACID CONCENTRATIONS IN RAZOR CLAMS AS A FUNCTION OF ELEVATION BETWEEN HIGH AND LOW TIDES AT KALALOCH BEACH WASHINGTON. **John C. Wekell*** and **Vera Trainer,** National Marine Fisheries Service, Northwest Fisheries Science Center, 2725 Montlake Blvd. East, Seattle, WA 98112; **Dan Ayres** and **Doug Simons,** Washington Department of Fish and Wildlife, 48 Devonshire Rd., Montesano, WA 98563.

Reported domoic acid levels in razor clams (*Siliqua patula*) in Washington State have been extremely variable and unpredictable, resulting in emergency closures of harvest areas in 1991, 1998, and 1999. This may be due to locational differences in clam toxicity. Information concerning variability in toxin levels relative to sampling location is important in developing a reliable sampling plan for managing domoic acid outbreaks. In November 1998, Kalaloch Beach in Washington State reported record levels of domoic acid in razor clams of about 300 ppm. Due to the relatively long retention time of this toxin in these clams, a resource survey at Kalaloch presented an opportunity for the study of domoic acid levels as a function of tidal elevation. From July 28–31, 1999 (during the summer low tides) six "east-west" transects were sampled at Kalaloch Beach, approximately 2 km apart. The eastern terminus of each transect was approximately 50 ft below the high tide mark and proceeded due west toward the water to the low tide mark, a total distance of approximately 300 to 450 feet. Samples of 10 razor clams were taken at each 50 ft interval. Clams were transported on ice to the Seattle laboratory, where they were individually measured, weighed, and shucked. The whole meats were individually homogenized, placed in containers, and frozen until analysis. Each clam was individually analyzed for domoic acid. Data is presented on the distribution of domoic acid both between transects (interspecific variability) and within (intraspecific variability) each transect.

VARIANCE IN AMNESIC SHELLFISH POISONING IN GEOGRAPHICALLY DISCRETE POPULATIONS OF RAZOR CLAMS (*SILIQUA PATULA*) IN BRITISH COLUMBIA. **J. N. C. Whyte,* N. G. Ginther,** and **L. J. Keddy,** Fisheries and Oceans Canada, Pacific Biological Station, 3190 Hammond Bay Road, Nanaimo, B.C., Canada, V9R 5K6; **R. Chiang,** Canadian Food Inspection Agency, 2250 South Boundary Road, Burnaby, B.C., Canada, V5M 4L9.

Domoic acid (DA) the cause of Amnesic Shellfish Poisoning is produced by *Pseudo-nitzschia* spp. DA retention in razor clams is significantly higher than in other Pacific bivalves, and provided a

means of diagnosing seasonal variance in DA producing blooms in clam areas. Major populations of razor clams are limited to the north coast of Graham Island (McIntyre Bay), the Queen Charlotte Islands, and Long Beach (Cox Bay) on the west coast of Vancouver Island. DA in clams from Cox Bay over a 2 year period varied from 3.5 ± 1.3–33.3 ± 6.9 μg/g, with higher toxicity following storms, which suggested advection of *Pseudo-nitzschia* from offshore. Toxicity in body tissues declined in the order of foot, siphon, gut (stomach and digestive system) and mantle (with adductor gill and gonad). However, the mantle always contained the highest percentage of the total toxin accumulated. Data analysis from Cox Bay clams indicated a clearance rate of 2.5 μg/g/d (r^2 = 0.7398). Toxicity in clams from McIntyre Bay during the same survey period ranged from 0.1–1.1 ± 0.3 μg/g, suggestive of infrequent formation of toxic *Pseudo-nitzschia* blooms in the north coast. Interestingly, 14 days after the conclusion of the 2 year survey toxicity increased to 31.6 μg/g, with the occurrence of a major *Pseudo-nitzschia* bloom. Decline in toxicity over the next year indicated a clearance rate of 1.9 μg/g/d (r^2 = 0.7202). A significant increase in toxicity in whole and edible tissue of clams from west to east along McIntyre Bay was considered to reflect increased on-shore catchment of *Pseudo-nitzschia* cells from the counter-current oceanographic gyre impacting the spit that extends into the eastern part of the Bay.

EXPERIMENTAL EXPOSURES OF BAY SCALLOPS TO CULTURES OF SUSPECTED HARMFUL MICROALGAE. **Gary H. Wikfors,* Jennifer H. Alix,** Milford Laboratory, Northeast Fisheries Science Center, NOAA Fisheries, Milford, CT 06460; **Sandra E. Shumway, Sara Barcia,** and **Julie Cullum,** Southampton College, LIU, Southampton, NY 11968; **Roxanna M. Smolowitz,** Marine Biological Laboratory, Woods Hole, MA 02543.

Widespread use of the term "Harmful Algal Bloom" begs the question: Harmful to whom? Molluscan shellfish have been recognized as vectors of microalgal toxins to human consumers for millennia, but detrimental effects of some microalgae upon the mollusks themselves have received less attention. As part of a larger study designed to investigate the role of grazing in the bloom dynamics of microalgae for which there is some evidence of grazing suppression, we conducted experimental exposures of bay scallops, *Argopecten irradians*, at several life-history stages (embryos, larvae, post-set, and juveniles) to a number of cultured microalgal strains. Microalgae investigated included: 1) dinoflagellates—two strains of *Prorocentrum minimum, Gyrodinium aureolum*, and *Gymnodinium splendens;* 2) a raphidophyte—*Heterosigma carterae;* and Prymnesiophytes—two strains of *Prymnesium parvum* and one of *P. patelliferum*. Scallop response variables measured included survival, growth, development, feeding behavior, and histopathology. Effects ranging from subtle and sublethal to acute toxicity were observed. The most dramatic, lethal effects were seen with a new strain of *Prorocentrum minimum*, collected by Dr. Patricia Glibert from a 1998 bloom in the York River, MD, and with a new strain of *Prymnesium parvum*, isolated by Dr. Robert Guillard from Boothbay Harbor, ME. In addition to limiting harvest of molluscan shellfish for human consumption, clearly harmful algal blooms have the potential to affect the population biology of molluscs themselves.

SHELLFISH BIOLOGY

THE BULBUS ARTERIOSUS OF THE CLAM *MERCENARIA MERCENARIA*: ANATOMY AND PHARMACOLOGY. **Lewis E. Deaton,* Bruce E. Felgenhauer,** and **Daniel W. Duhon,** Biology Department, University of Louisiana at Lafayette, Lafayette, LA 70504.

In bivalves, the hemolymph is pumped by a heart comprised, in general, of a ventricle and two auricles. Blood exits the heart via one or two aortae. In the quahog, *M. mercenaria*, there is a large swelling associated with the posterior aorta. This sac-like structure, the bulbus arteriosus, has a volume equal to that of the ventricle. The function of this tissue is unknown. We have investigated the anatomy, ultrastructure, and pharmacology of the bulbus. The wall of the bulbus consists of a spongy matrix of connective tissue interspersed with bundles of muscle. Neurons are also present; the axons are gathered into bundles. We also observed granulocytic hemocytes in the lumen and the wall of the bulbus. Unlike that of the ventricle, the lumen of the bulbus is largely devoid of trabeculae. The lumen of the bulbus is connected to that of the ventricle by the posterior aorta. This vessel continues as a tubular structure for at least half the length of the lumen of the bulbus before the aorta empties into the bulbus. The isolated bulbus arteriosus contracts tonically in response to 5-hydroxytryptamine, acetylcholine, and the molluscan neuropeptide FMRFamide. The threshold for these effects is about 10^{-7} M. Bioassays of acetone extracts of bulbus tissue on the ventricle of *M. mercenaria* show that the bulbus contains acetylcholine and FMRFamide. We injected ink into the lumen of the ventricle of *M. mercenaria in vivo* to observe the flow of hemolymph through the anterior and posterior aorta; the majority of the hemolymph ejected from the ventricle enters the anterior circulation. We conclude that the bulbus is probably involved in the regulation of the relative volumes of hemolymph delivered to the anterior and posterior aortae. Increases in the pressure of the hemolymph in the bulbus would constrict or collapse the poterior aorta. In addition, the presence of neurons in close association with the lumen of the bulbus suggests that it may also function as a neurohemal site. We did not, however, see any release of products into the lumen of the bulbus from neurons.

THE EFFECT OF LOW OXYGEN ON OYSTER SURVIVAL DURING REEF RESTORATION EFFORTS IN BON SECOUR BAY, ALABAMA. F. Scott Rikard* and **Richard K. Wallace,** Auburn University Marine Extension and Research Center, Mobile, AL 36615; **David Rouse** and **Imad Saoud,** Auburn University, Department of Fisheries and Allied Aquaculture, Auburn, AL 36849.

Low dissolved oxygen levels have been implicated in the decline of once-productive oyster reefs in Bon Secour Bay, Alabama. Since low dissolved oxygen often occurs near the bottom, it has been suggested that successful reef restoration might begin by increasing the height of the shell base above the existing bottom. Experimental 1 m^2 plots constructed of PVC rings and filled with oyster shell were established at Fish River Reef (depth 2.5 m) in Bon Secour Bay at two levels (20 cm and 40 cm) above bottom along with shell plots on bottom. Oysters held in mesh bags at these three levels survived and grew well the first year but suffered 100% mortality between the June 8, 1999 and August 13, 1999 sample dates. Oyster shell cultch from the three levels was sampled to analyze spat set and oyster growth. Shells sampled on July 13, 1999 had live oysters attached; 0.83, 1.10, 1.07 oysters/shell for the bottom, 20 cm and 40 cm levels, respectively. No live oysters were found on shells sampled on August 18, 1999. Remote water quality recording devices deployed continuously on Fish River Reef, recorded three periods of extended low dissolved oxygen (<0.5 mg/L) between the above sample dates. The longest instance was from July 16–21, 1999 for a period of approximately 130 hours. Similar, periodic low oxygen events probably prevent this and other reefs in the area from recovering to productive levels even when cultch is used to raise reef elevations 20–40cm.

USE OF DNA MARKERS TO DETECT DIFFERENTIAL LARVAL SETTLEMENT PATTERNS OF *MYTILUS EDULIS* AND *M. TROSSULUS*. Ellen L. Kenchington* and **Kenneth R. Freeman,** Bedford Institute of Oceanography, PO Box 1006 Dartmouth, Nova Scotia, Canada B2Y 4A2; **Scott P. Macquarrie,** Biology Department, Dalhousie University, Halifax, Nova Scotia, Canada B3J 4J1; **Shawn M. C. Robinson,** St. Andrew's Biological Station, St. Andrew's, New Brunswick, Canada.

The mussel aquaculture industry on the east coast of Canada is based on wild spat collection. The presence of *Mytilus trossulus* among commercially cultivated blue mussels (*M. edulis*) limits farm production, as the former has a lower yield and the thin shells are prone to breakage in the sorting equipment. Reports of simultaneous spawning of the two species and larval periods of equal duration combine to eliminate timing of collection as a means of avoiding *M. trossulus* at mixed-species farms. Field experiments were performed to determine spawning time and depth preference of the two species. Replicate polypropylene rope spat collectors were placed in the water during the last week of June and hauled and replaced at weekly intervals until August 7, and a fortnightly intervals thereafter through to October 16, encompassing the spawning period. The nuclear internal transcribed spacer (ITS) region of the ribosomal RNA gene array has been shown to distinguish these two species when digested with the restriction enzyme HhaI. Approximately 30 individual larvae were randomly picked from the collectors from each of three depths (1 m, 3 m, 5 m) on replicate ropes for each collection time. Following DNA extraction and PCR amplification of the ITS region and subsequent enzymatic digestion, the larvae were identified to species against known standards. The results indicate a clear statistically significant settlement depth preference for the two species, with *M. trossulus* favouring the shallower depths. By placing the collector ropes below 5 m at this site, growers could have increased the proportion of *M. edulis* collected by 100% and reduced the proportion of *M. trossulus* by 50%.

REPRODUCTIVE BIOLOGY OF PACIFIC OYSTERS: SOME ENIGMAS. Gretta Ó'Sullivan* and **Máire F. Mulcahy,** Department of Zoology and Animal Ecology, National University of Ireland, Lee Maltings, Prospect Row, Cork, Ireland.

Crassostrea gigas, a non-native species in Ireland, is cultured from hatchery produced spat, and was believed not to spawn under Irish conditions. Steele in 1996 and 1997 found that *C. gigas* spawned each year in Dungarvan Bay, but failed to spawn in Cork harbour even though temperatures and chlorophyll a levels were comparable at the two sites. She suggested that failure to spawn might be due to an environmental contaminant such as TBT. Furthermore Steele (1998) found only 0.002% hermaphrodites, whereas Sato (pers comm.) found between 23–68% hermaphrodites in samples examined from May to August in Japan.

This 12-month study examined the gonadal development and reproductive cycle of *C. gigas* in Dungarvan and Cork harbour, together with temperatures and chlorophyll a levels. The number of segments needed to establish accurately the gonadal variation between male, female and hermaphrodite was examined and found to be one. The possibility that TBT was responsible for the failure of Cork harbour oysters to spawn was examined by looking at shell and condition indices. It was found that oysters spawned again in Dungarvan, but failed to spawn in Cork harbour, though condition indices and gonadal maturatin were similar at both sites. However, shell index was lower in Cork harbour, supporting the hypothesis that TBT might be an inhibiting factor for spawning.

MOONLIGHT MADNESS AND LARVAL LAUNCH PADS: TIDAL SYNCHRONIZATION OF MOUND FORMATION AND HATCHING BY TANNER CRABS, *CHIONOECETES BAIRDI*. Bradley G. Stevens, NMFS, Kodiak Fisheries Research Center, 301 Research Ct., Kodiak, AK.

Using submersibles and ROV's, we observed female Tanner crabs forming dense aggregations of mounds during mating season each spring from 1991 to 1995, in 150 m depth in Chiniak Bay,

Kodiak, Alaska. From mid-April to June 1999, we investigated the relationship of mound formation to hatch timing and environmental factors on several fronts. A camera sled and ROV were used to monitor aggregation behavior; crabs started forming mounds by 13 April, and continued until June 1, forming the largest mounds ever seen. Female crabs brought into the lab, some captured from mounds, released larvae from 1 May to 6 June. Individual crabs required from 5 to 22 days (median 10) to release 14,000 to 226,000 larvae; the most released in a single day was 106,000. Embryonic heartrate increased steadily until hatching, offering a potential method for determining developmental stage. The median hatching date (17 May) coincided with the new moon, the highest spring tide in May, and a monthly reversal of mean current direction. There was no obvious correspondence between hatching or mound formation and Secchi disk depth or water temperature. Nor was there any significant difference in timing of hatching between crabs maintained in filtered or unfiltered seawater. We conclude that mound formation is associated with hatching, and is timed to occur during a period of high tidal current flow, though prior plankton blooms may be a partial cue. Mounds may serve as "larval launch pads" to facilitate escapement from the silty bottom and its boundary layer.

SHELLFISH HEALTH MANAGEMENT

ANTIMICROBIAL ACTIVITY IN CELL-FREE HEMOLYMPH OF OYSTERS AND MUSSELS. Robert S. Anderson* and **Amy E. Beaven,** Chesapeake Biological Laboratory, University of Maryland Center for Environmental Science, P.O. Box 38, Solomons, MD 20688.

The antimicrobial activity of sera from *Crassostrea virginica, C. gigas, Mytilus edulis,* and *Geukensia demissa* was tested using a laboratory propagated strain of the oyster parasite, *Perkinsus marinus,* and a bacterial species, *Bacillus megaterium.* The growth kinetics of *P. marinus* in the presence of bivalve sera were followed turbidometrically, and the cidal effects of exposures to standardized serum protein levels determined. Bactericidal activity was measured by determining the percent survivorship after serum exposure by the MTS/PMS assay. Activity of sera were compared after calculating EC_{50} values (μg serum protein per ml required to inhibit/kill 50% of the test microbes). Sera from local and Maine *C. virginica* had low, but detectable, anti-*P. marinus* activity ($EC_{50} \cong 1$–2 mg/ml), suggesting that exposure/infection was not a sole determinant of activity. Sera from *C. gigas* had no anti-*P. marinus* activity, although this species is reportedly less susceptible to this parasite than *C. virginica.* Both *M. edulis* and *G. demissa* sera had ~100- to 200-fold greater anti-*P. marinus* activity than *C. virginica.* Anti-*B. megaterium* activity was consistently recorded for all the *Crassostrea* species tested ($EC_{50} \cong 200$ μg/ml), as well as for *M. edulis* ($EC_{50} \cong 45$ μg/ml). No antibacterial activity was measured in *G. demissa* serum. Hemocyte extracts of *C. virginica* and the two mussels had higher anti-*P. marinus* specific activity than the corresponding sera. Hemocyte extracts of *C. virginica* and *M. edulis* had weaker anti-*B. megaterium* activity than the corresponding sera. Bivalve sera were fractionated by ultrafiltration to determine the MW of anti-*P. marinus* proteins. Unlike the oysters, *M. edulis* serum showed strong anti-*P. marinus* activity in the <10 kDa peptide fraction, suggesting the presence of defensin-like molecules. These data indicate antimicrobial agents show species-specific patterns of expression and activity in bivalves, some may be produced by hemocytes, and may partially determine resistance to infectious disease.

MANAGEMENT OF JUVENILE OYSTER DISEASE (JOD) IN MAINE. Bruce J. Barber,* Christopher V. Davis, Ryan B. Carnegie, and **Katherine J. Boettcher,** School of Marine Sciences, University of Maine, Orono, ME 04469.

Juvenile Oyster Disease (JOD) is a syndrome that affects juvenile oysters, *Crassostrea virginica* during the first growing season. Signs of JOD include reduced meat weight, uneven valve growth, and characteristic conchiolin deposits on inner valve surfaces. Since 1988, JOD has been responsible for cumulative mortalities of up to 96% in the Damariscotta River, Maine. Efforts to minimize the impact of this disease on commercial oyster production have involved both short-term and long-term approaches. Initial research revealed that mortality caused by JOD was inversely related to oyster size. Oysters with a mean shell height of 12.1 mm had a cumulative mortality of 56.2% while larger oysters (25.9 mm mean shell height) had a cumulative mortality of 13.6%. A subsequent study determined that mortality caused by JOD was seasonal in nature; cohorts placed in the river before June or after mid-August had cumulative mortalities <20% while those deployed between June and August had cumulative mortalities of 64–96%. Thus short term management strategies involve early spawning and deployment to achieve maximal size prior to the onset of disease. Longer term management has been accomplished through genetic selection. Selected oysters (Flowers F_3) had a cumulative mortality of 11.2% compared to 95.7% for unselected oysters. Ultimately, further management strategies will depend on the identification of an etiological agent. Recent experiments showed that oysters exposed to antibacterial agents had a lower cumulative mortality (55%) than control groups (81%). Further, bacteriological analysis revealed that a novel alpha-proteobacterium is numerically dominant in oysters exhibiting signs of JOD and not detected in healthy oysters. Challenge experiments with this suspect pathogen are ongoing.

DESCRIPTION OF AN UNUSUAL PARASITE IN PRAWNS, *PANDALUS PLATYCEROS*, IN BRITISH COLUMBIA, CANADA. **Susan M. Bower*** and **Gary R. Meyer,** Fisheries and Oceans Canada, Pacific Biological Station, Nanaimo, B.C. V9R 5K6, Canada.

A protozoa parasite, superficially similar to parasitic dinoflagellates, with large plasmodia and numerous trophonts occurred in up to 27% of the prawns from Malaspina Strait, British Columbia. Infections in most prawns were cryptic but of sufficient duration to affect secondary sexual characteristics and castrate the host. Cryptic infections consisted of large plasmodia containing numerous pleomorphic nuclei. Examination via electron microscopy revealed that in some areas of the plasmodium, the outer membrane was indistinct and the cytoplasm of the parasite appeared to coalesce with the cytoplasm of lysed haemocytes. The plasmodia invaded the haemal sinuses of all tissues and then broke up into trophonts with single nuclei. Prawns with gross evidence of infection (body discolouration, lethargy and haemolymph milky with a plethora of either spherical or discoid trophonts) rarely exceeded a prevalence of 2% of an infected population fished with traps. In a few prawns with mainly spherical trophonts, about 25% of the trophonts were dividing. The ultrastructure of nuclei containing mitotic figures consisted of a few condensed chromosomes attached by microtubules (spindle fibers) to centriole-like structures situated at a gap in the nuclear membrane. Nuclear division of trophonts in binary fission was unlike that described for a parasitic dinoflagellate (*Syndinium*). Also, detailed morphological examination did not reveal features characteristic of parasitic dinoflagellates (e.g., trichocysts in the cytoplasm and a flagellated stage). Thus, the taxonomic affiliation of the parasite in *P. platyceros* must be addressed using tools additional to morphological examination such as molecular analysis. Attempts to transmit the infection between prawns in the laboratory were unsuccessful.

INSIDE THE SHELL OF AN INTERTIDAL OYSTER: LIABILITIES AND BENEFITS? **L. E. Burnett*** and **C. S. Milardo,** Grice Marine Laboratory, University of Charleston, SC 29412.

When the oyster *Crassostrea virginica* is air exposed, it isolates itself nearly completely from the outside environment. The environment within the oyster shells change rapidly and dramatically. The degree of change depends on the ambient temperature and the microhabitat. An oyster in full sunlight becomes much hotter than those that are shaded. Tissues become hypoxic, but not anoxic, and acidic. Hemolymph O_2 pressure falls from 37 torr in an oyster in well-aerated water (155 torr = air saturation) to 10 torr during emersion. Po_2 never falls below 10 torr and the oyster depends entirely on anaerobic metabolism. Hemolymph pH falls as low as 6.0 at 35 °C due largely to the buildup of CO_2. These changes are similar to those that occur when an oyster is exposed to hypoxic water. Liabilities: low O_2 and separately low pH depress ROI production of oyster hemocytes. Although ROI production may not be bactericidal in oysters *per se* (Bramble & Anderson, 1999), bactericidal activity of oyster hemocytes under these conditions needs to be assessed. Benefits: the elevated CO_2 that occurs with emersion stimulates the metabolism of the parasite *Perkinsus marinus* and this may benefit the oyster in that the parasite directs more energy to respiration and less to growth and reproduction. This explains why infections of *Perkinsus* in intertidal oysters in the southeast are rarely very intense. (SC Sea Grant R/ER-14)

DISEASE DIAGNOSIS BY PCR: FOOLPROOF OR FOOLHARDY? **Eugene M. Burreson,*** Virginia Institute of Marine Science, College of William and Mary, Gloucester Point, VA 23062.

The polymerase chain reaction (PCR) is viewed by many as the ultimate diagnostic tool because of its extreme sensitivity and specificity. A positive PCR result is often interpreted as the unequivocal presence of a disease agent, and a negative PCR result is often interpreted as the unequivocal absence of a disease agent. However, the nature of PCR may result in false positives for disease diagnoses. A positive PCR does not necessarily mean that a viable disease agent is present because DNA may be isolated from samples with lysed or non-viable organisms. Two kinds of subsampling error potentially yield PCR false negatives—subsampling of uninfected tissue from a host for DNA extraction when infections are localized, and utilization of insufficient amounts of extracted host/parasite genomic DNA for PCR analyses. Replicate subsampling is recommended to minimize both types of error. At present, PCR diagnosis should be used in conjunction with standard techniques where possible. Nonetheless, PCR is extremely valuable for identifying known disease agents for which a gene sequence has been determined, especially when they occur in unexpected hosts. PCR is also extremely valuable when followed by sequencing for determining the phylogenetic position of undescribed parasites. More research is necessary comparing PCR and standard diagnostic techniques before PCR can be recommended as the method of choice for disease diagnosis.

SHELLFISH HEALTH MANAGEMENT: A SYSTEM LEVEL PERSPECTIVE FOR *PERKINSUS MARINUS*. **David Bushek,*[1,2] Jennifer Keesee,[1] Ben Jones,[1] Dave White,[2] Matt Neet,[1]** and **Dwayne Porter,[1,2,3]** [1]Baruch Institute, [2]Marine Science Program and [3]Department of Environmental Health Sciences, University of South Carolina, Columbia, SC 29208.

The oyster pathogen *Perkinsus marinus* has wreaked havoc on natural and cultured populations of the eastern oyster for more than half a century. Few management strategies have been developed to minimize *P. marinus*-induced oyster mortality and none have been effective. One reason may be a poor understanding of the processes that control parasite transmission. We present data from

three years of spatially intense seasonal monitoring of *P. marinus* infection intensities in two South Carolina estuaries. The data include El Niño, La Niña and normal rainfall years and indicate that physical processes related to transmission, namely water residence time and flushing rates, are primary determinants of infection intensity. Landscape-level anthropogenic impacts that alter these hydrological processes (eg., upland ditching and drainage, channel dredging, jetty construction, etc) may be more important factors in exacerbating oyster mortality problems from *P. marinus* than pollutants commonly associated with development. Shellfish health management can and should take advantage of these relationships in three ways: 1) via site selection for planting, cultivating and harvesting oysters, 2) for selecting sanctuaries and reserves, and 3) to identify potential management regulations and mitigation efforts for coastal development. To proceed, the principles of estuarine oceanography need to be more widely incorporated into the management of *Perkinsus marinus* and most likely many other shellfish pathogens.

HIGH PERFORMANCE OF *CRASSOSTREA ARIAKENSIS* IN CHESAPEAKE BAY. **Gustavo W. Calvo,* Mark W. Luckenbach,** and **Eugene M. Burreson,** School of Marine Science, Virginia Institute of Marine Science, College of William and Mary, Gloucester Point, VA 23062.

As native eastern oyster, *Crassostrea virginica*, stocks have declined throughout much of the mid-Atlantic seaboard of the United States interest in the potential of non-native oyster species to restore the fishery and ecological functions has grown. To examine the performance of triploid *C. ariakensis* in comparison with that of diploid *C. virginica*, oysters (n = 300, age = 2 years, mean shell height = 60–64 mm) were deployed in floating mesh cages at each of two replicate sites within low, medium, and high salinity regimes (respectively, <15‰, 15–25‰, >25‰) in Chesapeake Bay and the Atlantic Coast of Virginia. Over the 16 mo. evaluation period, from May 1998 to September 1999, *C. ariakensis* exhibited higher disease resistance and superior survival and growth than *C. virginica*. Final mean cumulative mortality was >80% for *C. virginica* and <20% for *C. ariakensis*. After 14 mo. of deployment, mean shell height of *C. ariakensis* at low, moderate, and high salinity sites, was respectively 96 mm, 125 mm, and 140 mm. In comparison, mean shell height of *C. virginica* was respectively 72 mm, 85 mm, and 75 mm. Baseline samples revealed no *P. marinus* and a 4% prevalence of *H. nelsoni* (MSX) in *C. virginica* and 12% prevalence of *P. marinus* and no MSX in *C. ariakensis*. In all subsequent samples, collected in August and October 1998, and in May, August and September 1999, prevalence and intensity of *P. marinus* infections were consistently higher in *C. virginica* than in *C. ariakensis*. During the second summer of disease exposure, prevalence in *C. virginica* was 100% at all sites whereas prevalence in *C. ariakensis* ranged form 0–28%. Only light infections were present in *C. ariakensis* whereas heavy infections were found in *C. virginica*. MSX was absent in *C. ariakensis* and present in *C. virginica*. This study demonstrated a high performance of adult *C. ariakensis* in the lower Chesapeake Bay and in the Atlantic Coast of Virginia.

DEVELOPMENT OF A PCR ASSAY FOR DETECTION OF *BONAMIA OSTREAE* IN FLAT OYSTERS, *OSTREA EDULIS*. **Ryan B. Carnegie,* Bruce J. Barber,** and **Daniel L. Distel,** School of Marine Sciences, University of Maine, Orono, ME 04469; **Sarah C. Culloty,** Department of Zoology and Animal Ecology, University College, Cork, Ireland.

Rapid and sensitive methods for the detection of shellfish pathogens are needed for effective disease management. Flat oysters (*Ostrea edulis*) infected with the microcell parasite *Bonamia ostreae* were used to develop a polymerase chain reaction (PCR) assay that is faster and more sensitive than standard histology. Genomic DNA was extracted from hemolymph of a Maine oyster and the gill of an Irish oyster. Using the PCR and primers tuned to protistan rDNA, a single, identical amplicon was obtained from both samples. This product was determined by BLAST search to closely resemble rDNA genes belonging to members of the Phylum Haplosporidia. A PCR reaction specific for this sequence was designed and used to assay hemolymph and gill tissue from 154 oysters scored for *B. ostreae* based on hemolymph smears (overall *B. ostreae* prevalence was 44.8%). A product presumed to be the *B. ostreae* sequence was generated in 100% of "heavily" infected oysters; 100% of "moderately" infected oysters; 84.6% of "lightly" infected oysters; 65.0% of "scarcely" infected oysters; and 61.2% of those scored "uninfected". No PCR product was detected, however, in a negative control composed of 19 juvenile *Crassostrea virginica* from Virginia. A positive PCR signal for *B. ostreae* in a high percentage of "uninfected" oysters does not necessarily represent spurious amplification. It is likely that most oysters in *B. ostreae*-enzootic areas harbor parasites, but at levels too low to be detected by standard cytological or histological methods. Indeed, closer histopathological examination of 26 of the above oysters found 81.5% to harbor *B. ostreae*, including 93.3% that tested positive for *B. ostreae* using PCR.

SEROLOGICAL AFFINITIES BETWEEN *PERKINSUS MARINUS* AND SOME PARASITIC DINOFLAGELLATES. **Christopher F. Dungan*** and **Rosalee Hamilton,** Cooperative Oxford Laboratory, Maryland DNR, Oxford, MD 21654; **David Bushek, Jennifer Cardinal,** and **Alan Lewitus,** Baruch Marine Field Laboratory, University of South Carolina, Georgetown, SC 29442.

Nucleotide sequences of several genes from the apicomplexan protozoan oyster pathogen *Perkinsus marinus* consistently suggest that its strongest phylogenetic affinities are with dinoflagellate taxa. To test for phenotypic evidence of this suggested taxonomic

linkage, antibodies to *P. marinus* known to have specificity for *Perkinsus* species, but not other protozoa, were used to immunostain free-living and parasitic dinoflagellate, and dinoflagellate-like, organisms. Among tested dinoflagellates, 3/28 free-living species (11%) and 6/7 parasitic species (86%) were labeled by antibodies to *P. marinus*. These results indicate the presence of shared antibody binding epitopes common to *P. marinus* and dinoflagellates, in general. Antibodies to *P. marinus* consistently labeled parasitic dinoflagellates infecting 6 crustacean hosts, but failed to label a dinoflagellate-like parasite of Spot prawns, *Pandalus platyceros*, from both Alaska, USA and British Colombia, Canada. The reciprocity of this antibody crossreactivity was tested by immunostaining the same suite of syndinean dinoflagellate parasites, the dinoflagellate-like Spot prawn parasite, and *P. marinus*, with antibodies against the *Hematodinium* sp. parasite of Norway lobster, *Nephrops norvegicus*. Anti-*Hematodinium* sp. antibodies reciprocally labeled *P. marinus* and the 6 *Hematodinium*-species infecting crustacea, but also failed to label the dinoflagellate-like Spot prawn parasite. Identical reciprocal cross-reaction patterns for antibodies to *P. marinus* and *Hematodinium* sp. from Norway lobsters, when each was tested against the same suite of pathogens, confirms the presence of shared antigenic epitopes among the apicomplexan oyster pathogen and parasitic dinoflagellates infecting six crustacean species, and supports an hypothesized phylogenetic affinity between these disparate protozoan taxa. Diagnostic immunoassays to differentiate parasitic *Perkinsus* and *Hematodinium* species must control or eliminate the identified crossreaction. The Spot prawn dinoflagellate-like parasite is clearly distinct from both *P. marinus* and *Hematodinium*-species parasites of other crustacean hosts.

AN EXAMINATION OF ECOLOGICAL FACTORS GOVERNING PLANKTONIC ABUNDANCE AND DISPERSAL OF *PERKINSUS MARINUS*. **Rebecca Ellin*** and **David Bushek,** Belle W. Baruch Institute for Marine Biology and Coastal Research, University of South Carolina, P.O. Box 1630, Georgetown, SC 29442.

Direct transmission of *Perkinsus marinus* to the Eastern oyster, *Crassostrea virginica*, occurs via the water column. The processes that influence planktonic transmission and dispersal remain poorly understood because few studies have attempted to examine the planktonic stages of *P. marinus*. For example, tidal influences on planktonic *P. marinus* across an oyster reef have never been examined and only a few studies have addressed the seasonal pattern of planktonic *P. marinus*. We hypothesize that tidal processes and the abundance of oysters govern the dispersal and transmission of *P. marinus* through the water column. To address these hypotheses, we are measuring changes in planktonic abundance of *P. marinus* across tidal cycles from the fronts and backs of four intertidal creeks in North Inlet, South Carolina. Oysters had been removed from two of these creeks enabling us to elucidate the role of oysters in the planktonic population dynamics of *P. marinus*. Five hundred milliliter water samples have been collected monthly from each location during neap tidal cycles since February 1999 (n = 24 per site). Samples are filtered, incubated in RFTM, and digested with NaOH to enumerate *P. marinus*. Preliminary examination of samples processed indicates an increase in *P. marinus* cells at slack high and low tides for all creeks. Furthermore, creeks with oysters exhibit higher *P. marinus* concentrations on flooding tides when compared to creeks without oysters.

BACTERIAL PATHOGENS, DISEASES AND THEIR CONTROL IN BIVALVE SEED CULTURE. **Ralph Elston,*** AquaTechnics/Pacific Shellfish Institute, PO Box 687, Carlsborg, WA 98324 USA; **Arthur Gee,** Dept. Biology, Pacific Lutheran University, Tacoma, WA 98447-0003; **Russell P. Herwig,** School of Fisheries, University of Washington, 3707 Brooklyn Ave. N.E., Seattle, WA 98105-6715.

Vibriosis is known as a disease of intensively cultured larval shellfish but bacterial pathogens cause significant losses in nursery cultures of juvenile bivalves. Typically, rod-shaped bacteria attach to externally oriented periostracum and subsequently invade juveniles through the valve closure and along the internal shell surface. Contact necrosis and sloughing of mantle epithelium results and, when bacteria have invaded sufficiently far along the mantle, they invade the still patent coelomic cavity of juvenile bivalves. A chronic form of the disease occurs less frequently. Detailed studies of invasive juvenile bacterial diseases are underway for the Pacific oyster (*Crassostrea gigas*), Kumomoto oyster (*Crassostrea sikamea*), geoduck clam (*Panope abrupta*), and other species.

Vibrio tubiashi, V. anguillarum, V. tapetis and *V. splendidus* have previously been reported as causative or associated with larval bivalve mortalities but there also appear to be significant unnamed vibrio-like pathogens of bivalve juveniles. Results of current studies to characterize pathogenesis and link disease types to bacterial species are underway, including identifying characteristics of the causative agents by morphological, physiological, nucleic acid and fatty acid analysis.

Bacterial pathogens enter culture systems via sea water, brood stock transport of in algal food cultures. They can be maintained on system surfaces and their growth augmented by dissolved organic substrates generated by algal cultures, external algal blooms, or metabolism of the cultured juveniles. Prevention and control strategies must include routine sanitation of system surfaces, water filtration, brood stock sanitation and maintenance of low dissolved organic levels. Antibiotics have been used in experimental settings but are not routinely used on production scale systems due to cost as well as risk of producing resistant strains. In the United States, there are no antibiotics licensed for general use on molluscan shellfish. A program to select and test probiotic strains of bacteria, as an alternative to antibiotic use, is underway and results to date will be presented.

LIFE HISTORY OF AN EXOTIC SABELLID POLYCHAETE, *TEREBRASABELLA HETEROUNCINATA*: INFLUENCE OF TEMPERATURE AND FERTILIZATION STRATEGY. **Carl A. Finley*** and **Carolyn S. Friedman,** California Department of Fish and Game and Bodega Marine Laboratory, P.O. Box 247, Bodega Bay, CA 94923 USA.

The California abalone aquaculture industry has been struggling to rid itself of an exotic sabellid, *Terebrasabella heterouncinata*, following its accidental introduction from South Africa in the late 1980s. The development of an effective management strategy is dependent upon the better understanding of the life history of this sabellid, including its generation time and whether it is capable of self-fertilization. In the present study, uninfested red abalone, *Haliotis rufescens*, were exposed over a 24 hr period to abalone with heavy infestations at 11.2, 15.6 and 20.9 °C; temperatures typically encountered in California. The larvae were subsequently observed as they developed to specific life stages: initiation of feeding, sexual maturation and the completion of their life history or the production of a motile, infestive, larva. Approximately 50% of the sabellids examined at 11.2, 15.6 and 20.9 °C had developed the ability to feed by day 6, 5 and 4 ($P < 0.001$), became sexually mature by day 83, 68 and 48 ($P < 0.001$) and had produced larvae by day 298, 165 and 111 ($P < 0.001$), respectively. In a separate study, uninfested abalone were exposed as above. Abalone with single infestations were held in individual container at 18 °C (single host and sabellid per container). This first, parental, generation was held in isolation until individuals self-fertilized to produce F_1 larvae. The F_1 larvae were subsequently isolated until individuals again self-fertilized, producing a second-generation, F_2 larvae. This research demonstrates that the life history and generation time of *T. heterouncinata* are significantly temperature dependent and that the products of self-fertilization are fully functional organisms.

FIELD TRANSMISSION STUDIES OF *HAPLOSPORIDIUM NELSONI* (MSX) USING SPECIFIC PRIMERS AND PCR TECHNOLOGY. **Susan E. Ford, Zhe Xu,** and **Gregory Debrosse,** Haskin Shellfish Research Laboratory, Rutgers University, Port Norris, NJ 08349.

Management of shellfish populations subjected to disease requires epizootiological data, including a knowledge of transmission. Incomplete understanding of transmission of the MSX disease agent, *Haplosporidium nelsoni*, is a critical barrier to managing affected oysters. Using molecular techniques that specifically detect *H. nelsoni*, we examined several aspects of the transmission question during a preliminary study in Delaware Bay. We monitored the presence of putative infective particles and documented subsequent infections—or lack of them—in larvae and juveniles in a land-based hatchery and nursery system, as well as in native oysters in the Bay. Neither eyed larvae nor 1-mm spat held in the hatchery in 1-μm filtered, UV-treated water, became infected, nor did juveniles held for an additional 5 weeks in the same conditions. Those held during the same period in an on-land nursery receiving raw bay water readily became infected. Positive reactions in the feces/pseudofeces of patently uninfected oysters, which we considered an indication of infective particles taken from the water, were prevalent throughout the Bay in the spring and early summer of 1999. They disappeared after midsummer as *Perkinsus marinus* (Dermo) infections appeared. The widespread distribution of putative infective stages and positive PCR signals in the tissues is in sharp contrast with the low prevalences of *H. nelsoni* detected in Delaware Bay oysters with standard histology during the past decade, suggesting that native oysters have become highly resistant to *H. nelsoni* infection development.

"*CANDIDATUS* XENOHALIOTIS CALIFORNIENSIS," A NEWLY DESCRIBED BACTERIAL PATHOGEN AND ETIOLOGICAL AGENT OF ABALONE WITHERING SYNDROME. **Carolyn S. Friedman,*** **Thea T. Robbins,** and **James D. Moore,** California Dept. of Fish & Game, Bodega Marine Laboratory, P.O. Box 247, Bodega Bay, CA 94923; **Jeffrey D. Shields,** Virginia Institute of Marine Science, Gloucester Point, VA 23062; **Karl B. Andree, Katherine A. Beauchamp, Dolores B. Antonio,** and **Ronald P. Hedrick,** Dept. of Medicine & Epidemiology, School of Veterinary Medicine, UC Davis, CA 95616.

Withering syndrome (WS) is a fatal disease of wild and cultured abalone, *Haliotis* spp., that inhabit the west coast of North America. Using a combination of morphological, serological, life history and genomic (16S rDNA) characterization, we have identified a previously undescribed bacterium observed in abalone with WS as a new member of the order Rickettsiales and propose the provisional status of "*Candidatus* Xenohaliotis californiensis". The Gram negative, pleomorphic bacterium is found within membrane-bound cytoplasmic vacuoles of abalone gastrointestinal epithelial cells. The bacterium is not cultivable on synthetic media or in fish cell lines and may be controlled by tetracyclines but not by chloramphenicol, clarithromycin, or sarafloxicin. Phylogenetic analysis based on the 16S rDNA of the bacterium places it in the α-subclass of the class Proteobacteria. We tested the hypothesis that this bacterium is the etiological agent of WS in two separate trials in which asymptomatic red or black abalone ± WS were administered a series of sham (3% saline) or OTC injections (21 mg/kg) over a 9 wk period. Both survival and feeding rates were higher in treated abalone relative to control animals ($p < 0.001$, $p < 0.023$ for red and black abalone, resp.). All red abalone and ~50% of the black abalone that received OTC survived, while ~40% of the red and 100% of the black abalone controls died during this time. These studies indicate that WS is caused by "*Candidatus* Xenohaliotis californiensis" and that losses can be minimized by administration of oxytetracycline. We have developed PCR and *in situ* hybridization tests for this bacterium and are in the process of developing an oral therapeutant. These tools will be useful in management of the disease and it's spread.

ISOLATION AND PRIMARY CULTURE OF EASTERN OYSTER HEMOCYTES. **Jerome F. La Peyre*** and **Yanli Li,** Department of Veterinary Science, Louisiana State University, Baton Rouge, LA 70803.

Most studies investigating the interactions of oyster hemocytes with pathogens rely on short term assays lasting only a few hours. The establishment of longer term hemocyte cultures (i.e., days to weeks) is needed to facilitate experimental manipulation. Such an *in vitro* system, for example, can be used to test the effects of various chemicals in promoting killing of pathogens by hemocytes. Our objectives for this initial study were to 1) compare five solutions for their abilities to inhibit clumping of oyster hemocytes during collection, 2) determine hemocyte sensitivity to antibiotics to be used in decontamination solution and culture medium, 3) optimize a basal medium to maintain hemocytes in primary culture & 4) test the effects of various chemicals on hemocyte adhesion either to promote the formation of hemocyte monolayers or to maintain hemocyte in suspension cultures. Our *in vitro* system was then evaluated by comparing *Vibrio parahaemolyticus* killing by hemocytes immediately after sampling and after culture for one week.

Using a variety of assays to measure oyster hemocyte mortality, metabolic activity and number, we found that: 1) Calcium and Magnesium-free oyster saline containing 0.5% EDTA was optimal for collecting hemocytes, 2) antifungal agents at concentrations generally recommended for cell culture were highly toxic to hemocytes, 3) increasing concentrations of carbohydrates, vitamins and amino acids were beneficial to hemocytes up to certain concentrations, 4) several chemicals including concanavalin A in basal medium and poly-D-lysine and fibronectin coated to culture vessels promoted the formation of hemocyte monolayers, 5) agarose coated to culture vessels was useful in maintaining hemocyte in suspension cultures unattached to culture vessels. Finally, *Vibrio parahaemolyticus* killing by hemocytes immediately after sampling and after culture for one week were comparable.

RETROVIRAL VECTOR-MEDIATED ONCOGENE TRANSFER TO CREATE *CRASSOSTREA VIRGINICA* CELL LINES. **James D. Moore,*** UC Davis Bodega Marine Laboratory, Bodega Bay CA 94923; **Viviane Boulo,** UMR 219, IFREMER-CNRS, Univ. Montpelier II, CP80, 34095 Montpelier, France; **Jane C. Burns,** Dept. of Pediatrics, UC San Diego, School of Medicine, La Jolla CA 92093-0830; **Carolyn S. Friedman,** Dept. of Medicine and Epidemiology, UC Davis School of Veterinary Medicine and California Dept. of Fish and Game, UC Davis Bodega Marine Laboratory, Bodega Bay CA 94923.

Management of shellfish disease requires sensitive tools for diagnosis and pathogen characterization. While tools developed for and applied in mammalian disease research are often quickly adapted for use with shellfish, one conspicuous exception is the failure to generate permanent cell lines from marine shellfish tissue. The absence of cell lines has particularly impeded research on viruses and obligate intracellular bacteria. We are attempting to create cell lines from tissues of the American oyster, *Crassostrea virginica* using a highly efficient method to integrate oncogenes into the genome of cells in primary culture. Heart tissue primary cultures were established in 24-well plates after enzymatic digestion of aseptically dissected heart tissue. Cells were then infected with replication-defective retroviral vector(s) containing viral promoters driving expression of the gene(s) of interest. These retroviral vectors contain the envelope glycoprotein of vesicular stomatitis virus that binds to phospholipid components of the cell membrane, thus allowing entry of the virus particle into a wide range of cell types. Conditions for gene transfer and expression were optimized using a vector construct containing the luciferase reporter gene, and assaying luciferase activity of primary cultures at 72 h post-infection with a scintillation counter in single photon mode. Luciferase activity, as a measure of infection efficiency, was linearly related to vector concentration from 5×10^3 to 8×10^5 cfu/well. Addition of the vector at the time of plating resulted in higher activity than addition at later time periods. Luciferase activity increased by centrifugation of plates (1000 g, 30 min), but was reduced by addition of *C. virginica* hemolymph to the medium during infection. Heart primary cultures are now being infected under optimal conditions with retroviral vectors encoding the SV40 large T antigen and ras oncogenes. Cultures are being monitored for morphological changes and replicative activity. Supported by the National Sea Grant College Gulf Oyster Industry Program through California Sea Grant College Grant No. NA86R60073.

DISTRIBUTION AND PREVALENCE OF BITTER CRAB SYNDROME IN SNOW (*CHIONOECETES OPILIO*) AND TANNER (*C. BAIRDI*) CRABS OF THE BERING SEA, 1988–1996. **J. Frank Morado,*** National Marine Fisheries Service, Alaska Fisheries Science Center, Resource Assessment & Conservation Engineering Division, 7600 Sand Point Way NE, Seattle, WA 98115-0070; **Theodore R. Meyers,** Alaska Department of Fish & Game, Commercial Fisheries Management and Development Division, Fish Pathology Section, PO Box 25526, Juneau, AK 99802-2000; **Robert S. Otto,** National Marine Fisheries Service, Alaska Fisheries Science Center, Kodiak Laboratory, PO Box 1638, Kodiak, AK 99615.

Bitter crab syndrome (BCS) is a fatal disease of crustaceans that is caused by a parasitic dinoflagellate of the genus *Hematodinium*. Between 1988 and 1996, hemolymph samples from 14,359 Eastern Bering Sea (EBS) Tanner crabs, *Chionoecetes bairdi* (N = 5180) and *C. opilio* (N = 9184) were examined for the presence of a parasitic dinoflagellate, *Hematodinium* sp., the causative agent of Bitter Crab Syndrome (BCS). For this time period, total

prevalences of BCS in *C. bairdi* and *C. opilio* were 1.87% and 3.57%, respectively. In *C. bairdi*, prevalences from yearly random samples ranged from 0% in 1989 and 1994 to 5.68% in 1996. Infections in both males and females were highest in 1996, reaching 9.93% in females and 2.74% in males; however, overall *Hematodinium* prevalences were only slightly more elevated in females (1.93%) than males (1.65%). For *C. opilio*, yearly random sample prevalences ranged from 0.30% in 1994 to 8.45% in 1988. Highest *Hematodinium* prevalences in *C. opilio* were observed early in the survey; in 1988 during which male and female infection prevalences were 7.62% and 10.00%, respectively. Overall parasitic prevalences in *C. opilio* were more elevated in females (4.23%) than males (3.23%).

BCS infections in both *C. opilio* and *C. bairdi* were most common in the Bering Sea at latitudes above 57°N. In general, infection prevalences in *C. opilio* increased with increase in latitude with prevalences of 50–80% common in Norton Sound and west of St. Lawrence Island. Despite the fact that prevalences were generally lower in the Chukchi Sea than in Norton Sound and west of St. Lawrence Island, a greater percentage of sampled stations were positive for BCS in the Chukchi Sea. For *C. bairdi*, infections were rare in the Eastern Bering Sea, and increased only slightly along the shelf edge west and north of the Pribilof Islands.

For both *C. opilio* and *C. bairdi*, infections were more common in small crab less than 60 mm; after which, prevalences remained low. In *C. bairdi*, the highest infection rates were observed in 20 mm crab attaining levels of 62.5% in males and 65% in females. In *C. opilio*, highest prevalences were observed at 35 mm with little difference in prevalence between males and females.

EFFECT OF A LYTIC PEPTIDE AND PROTEASE INHIBITORS ON *PERKINSUS MARINUS* IN INFECTED HEMOCYTES OF EASTERN OYSTERS. **Amy D. Nickens*** and **Terrence R. Tiersch,** Aquaculture Research Station, Louisiana State University Agricultural Center, Baton Rouge, LA 70820; **Jerome F. La Peyre,** Department of Veterinary Science, Louisiana State University, Baton Rouge, LA 70803.

In previous work, we found that a synthetic lytic peptide, phor-21, killed *P. marinus* cells *in vitro* at concentrations that were not lethal to oyster hemocytes. The efficacy of phor-21 was reduced however by extracellular proteases of *P. marinus*. Further study revealed that certain protease inhibitors, such as chymostatin and potato chymotrypsin-1 (PCI-1), protected phor-21 against the proteases of the parasite. In addition, each protease inhibitor was found to suppress the propagation of *P. marinus in vitro*. Therefore, we proposed that a combination of the lytic peptide and a protease inhibitor may be effective in eliminating *P. marinus* from eastern oysters. The objective of this study was to determine the effects of the lytic peptide and protease inhibitors on *P. marinus* in naturally infected hemocytes.

Hemocytes were collected from *P. marinus* infected oysters and monolayers were established in basal medium. The hemocyte monolayers were then exposed to phor-21, chymostatin, PCI-1 or a combination of phor-21 and a protease inhibitor. Hemocytes in basal medium alone or with DMSO (i.e., chymostatin solvent) were used as control. The number of *P. marinus* in hemocytes at the beginning of the experiment and after three days in culture was determined by counting the number of hypnospores after incubation in modified Ray's fluid thioglycollate medium. Preliminary results indicate that a combination of phor-21 and chymostatin, chymostatin alone and a combination of phor-21 and PCI-1 were most effective in reducing the number of parasites in our *in vitro* system.

MOLECULAR ANALYSES OF A PARASITE IN PRAWNS (*PANDALUS PLATYCEROS*) FROM BRITISH COLUMBIA, CANADA. **Kimberly S. Reece*** and **Eugene M. Burreson,** Virginia Institute of Marine Science, The College of William and Mary, Gloucester Point, VA 23062; **Susan M. Bower,** Fisheries and Oceans Canada, Pacific Biological Station, Nanaimo, B.C. V9R 5K6, Canada; **Christopher F. Dungan,** Cooperative Oxford Laboratory, Oxford, MD 21654.

A parasitic protozoan that appears morphologically similar to dinoflagellates has been found in the northeast Pacific Spot prawn, *Pandalus platyceros*. However, polyclonal antibodies raised against the oyster parasite *Perkinsus marinus* that have been shown to cross-react with several crustacean-parasitic dinoflagellates did not bind to this parasite. Muscle and hemolymph tissue samples from four infected prawns were obtained from Susan Bower (Pacific Biological Station, British Columbia, Canada) and DNA was extracted for nucleotide sequence analyses. Actin and small subunit ribosomal RNA (SSU rRNA) gene sequences were obtained from all the DNA isolations by amplification using "universal" primers in the polymerase chain reaction. Two distinct classes of sequences were obtained for each gene. One class showed high similarity to available crustacean sequences and was presumed to be from host DNA. In BLAST searches of GenBank with the second class of sequences, the highest match scores were to protozoan sequences. Two different DNA probes for *in situ* hybridizations were designed based on unique regions of the SSU rDNA sequence presumably from the parasite. Both probes hybridized specifically to parasite cells in histological sections of infected prawn tissue and did not hybridize to sections from other invertebrates infected with parasitic dinoflagellates or haplosporidians. Phylogenetic analyses based on the second class of sequences were done with data sets of more than 50 protozoan actin and SSU rRNA gene sequences. Results of parsimony analyses with both genes consistently grouped the prawn parasite with members of the phylum Haplosporidia, not with the dinoflagellates. These results confirm that two separate gene sequences from the parasite have been amplified from infected prawn DNA extracts, and strongly suggest that this parasite has haplosporidian affinities.

IDENTIFICATION OF A PROTOZOAN PARASITE IN THE AMERICAN LOBSTER, HOMARUS AMERICANUS, FROM LONG ISLAND SOUND. **Spencer Russell,* Kristen Hobbie, Tom Burrage, Claudia Koerting, Sylvain De Guise, Salvatore Frasca Jr.,** and **Richard A. French.** University of Connecticut, Dept. of Pathobiology, 61 North Eagleville Rd. U-89, Storrs, CT 06269.

Mortalities of the American lobster, *Homarus americanus,* in Long Island Sound have severely increased and as a result is critically damaging the regional lobster industry. Necropsies were performed on 75 individual lobsters collected from six different locations in Long Island Sound. Gross observations observed in 'sick' lobsters included a pink discoloration to the ventral surface of the abdomen (tail meat) and lethargic/limp behavior. An associated coagulopathy of hemocytes is also observed in affected lobster. Initial bacteriology findings include isolation of *Vibrio* spp. and spirochetes. No *Aerococcus* have been isolated to date. Histologic examination has been conducted on various tissues, including heart, gill, hepatopancreas, antennary glands, intestine, muscle, exoskeleton, eyes, antennae, and central nervous system. The histopathology is consistent with a systemic inflammatory disease affecting multiple tissues but primarily the nervous system. Associated with lesions is a protozoan parasite morphologically characterized as an amoeba, tentatively paramoeba sp.

COMPARATIVE EVALUATION OF THE MULTIPLEX PCR WITH CONVENTIONAL DETECTION METHODS FOR *HAPLOSPORIDIUM NELSONI* (MSX), *HAPLOSPORIDIUM COSTALE* (SSO), AND *PERKINSUS MARINUS* (DERMO) IN THE EASTERN OYSTER, *CRASSOSTREA VIRGINICA*. **Spencer Russell, Soledad Penna,** and **Richard A. French,** University of Connecticut, Dept. of Pathobiology, 61 North Eagleville Rd, U-89, Storrs, CT 06269.

Presently, the monitoring of cultured oyster populations for pathogens is infrequent due to the dependence on traditional, time consuming diagnostic assays. A multiplex polymerase chain reaction (MPCR) has been developed which rapidly detects the protozoan parasites, *Perkinsus marinus* (Dermo), *Haplosporidium nelsoni* (MSX) and *Haplosporidium costale* (SSO), which infect the cultured oyster, *Crassostrea virginica.* Conventional diagnostic methods (histopathology and Ray/Mackin fluid thioglycollate assay) for *H. nelsoni, H. costale* and *P. marinus* respectively we compared and evaluated with the MPCR. Ninety-one adult oysters were collected from randomly selected beds in Westport, CT, (n = 37) and Milford, CT (n = 54) and subjected to all three assays. The Ray/Mackin assay detected *P. marinus* infections in 59 of 91 (64%) oysters and MPCR revealed infections in 73 of 91 (80%) oysters. Histological examination detected 37 of 91 (40%) oysters infected with *Haplosporidium* plasmodia. The MPCR was able to differentiate between the two *Haplosporidium* plasmodia, detecting 9 of 91 (10%) oysters infected only with *H. nelsoni*, 37 of 91 (40%) oysters with only *H. costale,* and 32 of 91 (35%) oysters with mixed infections of *H. nelsoni* and *H. costale.* These results indicate the MPCR is a more sensitive assay for the detection of *P. marinus* and is able to detect and differentiate between the two *Haplosporidium* species. This would suggest that the MPCR can be useful at low infection intensity by being able to detect pathogens, based on pathogen DNA concentrations as low a 10 fg., for *H. nelsoni* and 1 pg. for both *H. costale* and *P. marinus.*

RESULTS OF QPX FIELD STUDIES. **Roxanna Smolowitz,* Ernest Marks,** and **Chris Brothers,** Marine Biological Laboratory, Woods Hole, MA; **Dale Leavitt** and **Bruce Lancaster,** Woods Hole Oceanographic Institution, Woods Hole, MA.

Studies of QPX, (Quahog Parasite Unknown) begun in October, 1997, are presently being conducted in Provincetown and Duxbury, MA. Spawn from three parentage groups were planted in experimental plots, 10 ft × 10 ft. Parentage profiles of the three hard clam (*Mercenaria mercenaria*) stains were: 20% wild/80% notata (mixed parentage), 100% notata and 100% wild parentage. Three types of management methods were used to attempt reduction of the disease occurrence and severity in the experimental clams. Hard clams (mixed parentage only) deployed in Duxbury, MA were planted in previously infected plots that had laid fallow for one year before planting. Clams in Provincetown were planted in three locations. One location was in a lease which contained residual infected clams and represented a positive control plot. The remaining two locations represented plots in which possible management methods would be tested. The first of these was located in a heavily infected lease, adjacent to the positive control plot. However, this experimental plot's sediment was hydraulically turned and limed one month before planting. The second of the two types of managed plots was established in an area that had never been aquacultured before (naive sediment).

As of the fall, 1999 sampling period, the percentage of mixed parentage animals grossly positive in Duxbury was high (38% contained mantle nodules), although no significant mortality was noted. Based on previous experience, it is expected that mortality will occur during the spring of 2000. As of fall, 1999, the mixed parentage clams collected from Duxbury were significantly larger than the mixed parentage Provincetown clams. It is therefore unlikely that decreased food quantity/quality of a bay or estuary is a significant factor (stressor) in the positive development of the disease. Additionally, allowing land to lie fallow for a year before planting does not appear to prevent the infection of subsequently planted hard clams.

No nodules were noted grossly in any of the hard clam strains sampled in Provincetown in the fall of 1999. The possibility of strain resistance to QPX infections cannot be evaluated till subsequent samples are collected.

DNA-BASED MOLECULAR DIAGNOSTICS FOR THE HARD CLAM PARASITE QPX (QUAHOG PARASITE UNKNOWN). **Nancy A. Stokes,* Lisa M. Ragone Calvo,** and **Eugene M. Burreson,** Virginia Institute of Marine Science, College of William and Mary, Gloucester Point, VA 23062.

The protistan parasite QPX (Quahog Parasite Unknown) has been reported in populations of cultured hard clams *Mercenaria mercenaria* in New Brunswick and Prince Edward Island, Canada and in Massachusetts, New Jersey, and Virginia, USA. Aspects of the life cycle, taxonomy, and epizootiology of QPX are poorly understood. To date the sole diagnostic method for the pathogen is histological examination of tissue sections. Development of additional diagnostic tools in the form of DNA probes for in situ hybridization and primers for polymerase chain reaction (PCR) amplification will enable researchers to better understand QPX and its disease process.

Genomic DNA was isolated from QPX cultured cells and the small subunit ribosomal DNA (SSU rDNA) was PCR amplified and sequenced. The SSU rDNA sequences of QPX, *M. mercenaria*, and several members of the phylum Labyrinthomorpha, which is currently the tentative placement of QPX, were aligned and regions specific for QPX were located. DNA-based diagnostic tools were designed based on two of these specific regions. The DNA probe specifically hybridized to QPX cells in tissue sections of infected clams collected from Virginia and Massachusetts. The PCR primers specifically amplified a 665 bp region of the QPX SSU rDNA and were able to detect the QPX target sequence from infected clam genomic DNA or from 1 fg of cloned QPX SSU rDNA.

EFFECTS OF PERKINSOSIS ON THE CLAM *RUDITAPES DECUSSATUS* INDUSTRY OF GALICIA (NW SPAIN). **Antonio Villalba,* Sandra M. Casas, María J. Carballal,** and **Carmen López,** Centro de Investigacións Mariñas, Aptdo. 13, Vilanova de Arousa 36620, Spain.

A research program is being developed to evaluate the potential effect of perkinsosis in clam *Ruditapes decussatus* populations of Galicia. Temporal patterns of variation of parasitization by *Perkinsus atlanticus* are being studied. Samples have been taken monthly, since 1996, from a bed with high perkinsosis prevalence. Clam size (age) and infection intensity were significantly correlated. No infection was detected in clams smaller than 20 mm in length. Every clam longer than 48 mm was infected. A seasonal pattern of infection intensity variation was observed in the 4 study years. High values were found in spring and late summer—early autumn. The lowest values were recorded in winter.

Influence of the parasite on clam growth is being studied through the effects on clam's energetic physiology. Experiments were performed to estimate respiration and clearance rates of 50 clams, at 15 °C temperature and 35 ppt salinity. Then, clams were processed for disease diagnosis. Both physiological rates were lower only in heavy infections. New experiments will be performed at warmer conditions. Consistently, a significant decrease of condition was detected only in heavy infections, both in ripe and resting gonad periods. Nevertheless condition decrease was not severe in any case.

Influence of temperature and salinity on zoosporulation and viability of free life stages of *P. atlanticus* was evaluated. Prezoosporangia were exposed to different salinity conditions (2.5, 5, 10, 15, 20, 25 and 35 ppt), at 2 temperatures (19 and 28 °C). In other experiment, prezoosporangia were held at different temperatures (4, 10, 15, 22, 28 and 32 °C), at 30 ppt salinity. Zoosporulation occurred in wide ranges of temperature (15–32 °C) and salinity (10–35 ppt). The optimum values were 19–28 °C and 25–35 ppt. The highest temperatures in Galician rias (20–22 °C) are within the optimum range for zoosporulation. Some prezoosporangia survived up to 66 and 129 days at 4 and 10 °C, respectively. Prezoosporangia that have been held at 4 and 10 °C for 16 and 67 days, respectively, and subsequently transferred to 22 and 28 °C, gave rise to zoospores. Thus, prezoosporangia could overwinter without zoosporulating, and would produce zoospores in favourable conditions. Zoospores survived for more than 20 days, at 28, 20, 15 and 10 °C in an experiment to test longevity.

DESIGN CRITERIA FOR MICROALGAL FEEDS PRODUCTION SYSTEMS, AND THE GRAMPS EXPERIENCE. **Gary H. Wikfors*** and **Barry C. Smith,** Milford Laboratory, Northeast Fisheries Science Center, NOAA Fisheries, Milford, CT 06460; **Loy Wilkinson,** Coastal BioMarine, Bridgewater, CT 06752.

"We can't afford to feed them!" The "them" of this exclamation are post-set molluscan shellfish, and the "we" are nearly every shellfish farmer who needs to sell shellfish for more than it costs to grow them. This universal paradigm drives the nursery strategy of "raw-water" rearing of post-set shellfish seed. Consequences of raw-water nursery culture are seasonal (temperature) constraints on seed production; exposure of young animals to environmental stresses, predators, and disease; and a loss of control over the nutritional input to the animals. Land-based nursery culture of molluscs under controlled conditions would solve most of these limitations if the cost of producing feeds—cultured algae or prepared diets—is lowered substantially. Current hatchery and nursery microalgal feed production systems incur costs in the range of $100–400 per dry kilogram of algal biomass. At the high end of this range, a farmer would need to sell bay scallop meats for about $750 per pound; thus, a decrease in the range of two orders of magnitude for microalgal feeds is needed for economical, controlled shellfish rearing.

Fundamental knowledge of microalgal culture requirements exists; therefore, design criteria for new microalgal feeds production systems must focus further on economics. A simple, economic analysis of using artificial light as the algal energy source indicates that ~99% of the cost is in electricity for the lights. Accordingly, use of solar energy can accomplish a cost reduction of one order of magnitude, but only if algal cultures can be sustained in the varying energy (light and temperature) field of natural solar cycles. A new facility has been built at the Milford Laboratory—the Greenhouse for Research on Algal Mass Production Systems (GRAMPS)—to address the challenges of growing microalgal aquaculture feeds in the undependable sunlight of the northeastern US. Using familiar, simple culture containers (kalwall tubes and open tanks), we are applying computer automation, process-control loops, and other cross-field technologies from contemporary industries, to microalgal culture on a pilot scale (ca. 20,000 liters production per day). GRAMPS successes and remaining challenges will be described. An economic analysis of GRAMPS operation indicates production costs in the range of $40 per dry kilogram of algal biomass, with expectation of 50% savings with optimization of control systems. This production cost appears to enable extended, land-based nursery culture of shellfish seed, but not yet grow-out of shellfish to market under controlled conditions.

TECHNOLOGICAL ADVANCES IN MOLLUSCAN AQUACULTURE

THE USE OF PROBIOTIC TECHNIQUES FOR CONTROLLING BACTERIAL DISEASES IN MARINE INVERTEBRATE HATCHERIES. A. O. Alabi, Island Scallops Limited, 5552 West Island highway, Qualicum Beach, B.C. V9K 2C8. Canada.

One of the difficulties encountered in commercial invertebrate hatcheries has been the poor larval survivals attributed to attack by opportunistic bacteria. A wide range of methods are therefore employed to limit and reduce the number of bacteria occurring in hatchery water supplies and rearing systems: Chemotherapeutants depend on a host having a higher tolerance threshold level to the substance than the target organism. However, these differences are often marginal and depend on the physiological state of the larvae. Bacterial resistance has also been reported in response to widespread and indiscriminate use of antibiotics. Other pre-treatment methods frequently used are filtration, ultra-violet (UV) light irradiation and ozonation. All these methods aim to reduce or eliminate bacteria in the water. However, disinfection or partial sterilization of sea water appears to encourage the selective development of bacterial communities which differ from those found in natural sea water.

The onset of bacterial diseases has usually been attributed to environmental changes which favor the development of excessive levels of a particular pathogen.

Obtaining control of the microbial environment of larval rearing systems should therefore permit increased manipulations of the bacterial flora and lead to increased larval survival. Such control may be obtained by maintenance of balanced populations of bacteria and by the use of defined probiotics. This study details results obtained in the successful commercial scale production of crustaceans, bivalves and echinoderms using these techniques. Potential shortcomings in the use of these methods are also discussed.

RECENT DEVELOPMENTS IN MOLLUSC HATCHERY TECHNIQUES. John Bayes, Seasalter Shellfish Company, Quercus, Willow Road, Whitstable, CT5 3DW, England.

Low impact and environmentally and economically viable algae systems are described in detail including their installation, operation and management in climates world-wide, and with particular reference to temperate regions such as are found in the West coast of America and Canada. Diet selection and control including reference to probiotic bacteria populations within the algae culture systems are described. In addition, water management for larvae and post-set juveniles to ensure maximum growth and survival is discussed and will include cost benefit analyses of continuous flow systems compared to batch cultivation.

RECIRCULATION SYSTEM DESIGN FOR SHELLFISH WET STORAGE OR DEPURATION. S. Chen,* B. B. Saucier, J. S. Zhu, and **E. Durfey,** Department of Biological Systems Engineering, Washington State University, Pullman, WA 99164 USA.

Research and development of new technologies that are applicable to shellfish operations will strengthen the competitiveness and enhance the sustainability of the shellfish industry. This presentation summarizes the findings of a study on optimal design of recirculating systems for shellfish depuration and wet storage. The project was funded by the Saltonstall-Kennedy Fisheries Research and Development Grant Program and conducted with collaboration from Taylor Resources, Inc. of Shelton, Washington. The use of water recirculating technology to wet-storage and depuration has the advantage of being cost effective, environmentally sound, and location independent, while meeting sanitation and other regulatory requirements. Major research results to be presented cover three major areas related to recirculating system design, including waste excretion, biofiltration, and disinfection. The research obtained quantitative information on waste generation from Manila clams and indicated that temperature was a major factor determining the excretion rate of total ammonia, total Kjeldahl nitrogen, and biochemical oxygen demand. Equations were developed for estimating waste excretion under different conditions. The purpose of biofiltration in a recirculating system is to convert ammonia to

less toxic nitrate through biological nitrification. The biofiltration study demonstrated that there was a very high nitrification potential for the biological filters even at a relatively low (10 °C) temperature. A nitrification potential of 1000 mg of ammonia nitrogen removal per square meter per day can be achieved for a total ammonia concentration of 2 mg/l in the water. The study also demonstrated that UV disinfection units were very effective in destroying coliform bacteria. The most important factors affecting UV disinfection efficiency were the UV transmittance of the water and the flow rate through the UV unit.

TRIPLOID PRODUCTION OF *MYTILUS EDULIS* IN PRINCE EDWARD ISLAND. **John W. Brake*** and **Jeffrey Davidson,** Atlantic Veterinary College, University of Prince Edward Island, and **Jonathan Davis,** Baywater, Inc. 15425 Smoland Lane, Bainbridge Island, WA 98110 USA.

The mussel aquaculture industry in Prince Edward Island (PEI), Canada is a well established major contributor to the island economy. PEI mussel production in 1998 was approximately 12,500 MT, with farm gate value exceeding $15.1 M (Can) and export value exceeded $30 M (Can). The industry supports over 1250 full and part time jobs.

The production of non-reproducing harvestable triploid mussels has been investigated for harvesting during the spawning season, when product quality and shipping are both problems, allowing the marketing of a high quality product year round. Many species have been produced and reared successfully as triploids and the production of triploid Pacific oysters (*Crassostrea gigas*) is currently extensively practiced in the Pacific Northwest. Identified methods of triploid induction (used at different levels or in combinations) in shellfish include temperature and/or pressure shocking and the use of chemicals such as caffeine, cytochalasin B, or 6-dimethylaminopurine.

The mussel industry has recognized the potential of harvesting triploid mussels during the spawning season. The objective of this study is to elucidate the optimal triploid induction methods for commercial use in PEI by the use of a matrix of previously identified triploidy induction methods. These combinations of methods are ranked by % induction and % survivorship, as well as feasibility in order to determine the best method. To date, inductions trials with a % triploid induction of >90% have been obtained, however, survival has been lower than expected. A new attempt to investigate the exact treatment timing window is being investigated in an attempt to improve survivorship. This method involves epifluorescent microscopy and the timing of treatment based upon the chromosome separation rather than polar body formation. The current methodology being used for triploid induction optimization and successful growth trials will be discussed along with some of the results obtained to date.

APPLICATION OF A SHELLFISH SCIENCE CLUB MODEL IN PUGET SOUND, WASHINGTON. **Daniel P. Cheney,*** Pacific Shellfish Institute, 120 State Avenue NE #142, Olympia, WA 98501; **John L. Pitts,** Bellwether Consulting, 3881 Leland Valley Rd. W., Quilcene, WA 98376.

The Pacific Shellfish Institute (PSI) provided training and facility development for science and vocational teachers in schools and tribal communities throughout the greater Puget Sound region to apply a shellfish model developed for the Quilcene-Brinnon Schools Shellfish Science Club, Quilcene, Washington. The program and curriculum of this model were designed to teach high school students how to farm shellfish, maintain water quality and habitat, and utilize the scientific method in resource conservation. Winner of the President's National Environmental Education Award, the model combines community education with a "junior achievement", entrepreneurial incentive for students, using farmed shellfish at local fairs and festivals. The introduction of water quality education for K-12 students and the establishment of a working relationship with local shellfish farmers moves the classroom into the field. PSI initially extended the concept to schools and tribes through a series of workshops. Two high schools were identified with staff and facilities suitable for the program. Teachers and students were introduced to a variety of shellfish culture concepts. These included: a) classroom instruction; b) population assessments and surveys on a commercial oyster culture site; c) farm tours to view shellfish polyculture (clams, oysters, geoducks); d) shellfish hatchery and processing plant field trips; and e) geoduck farming techniques and research site sampling. PSI is continuing to work with both schools to assist them with technical information, shellfish bed management, and coordination with shellfish farmer mentors. This project was supported by a grant from the Puget Sound Water Quality Action Team, Public Involvement and Education Fund.

MANIPULATION OF THE CEMENTING PROCESS OF THE PURPLE-HINGE ROCK SCALLOP, *CRASSADOMA GIGANTEA*. **Carolynn S. Culver,* John B. Richards,** and **Henry M. Page,** Marine Science Institute, University of California, Santa Barbara, CA 93106.

Culture of the purple-hinge rock scallop, *Crassadoma gigantea* (formerly *Hinnites multirugosus*) is of interest to many West Coast aquaculturists. However, grow-out techniques used to culture other bivalve species are not directly suitable for rock scallops. In contrast to other species, rock scallops end their free swimming phase and usually attach permanently to hard substrata. Permanent attachment is problematic for culturists. The shell becomes very irregular during growth, conforming to the substrate topology. This irregular shell shape, in addition to its firm attachment, causes harvesting and marketing problems. Damage to the product and culturing gear often results during removal of the attached scallop, reducing product value and increasing production costs.

Because of the difficulties associated with scallop attachment, economically feasible culture will depend on development of methods for control or manipulation of the cementing process. We have begun studies to identify biological and physical factors affecting this process. Preliminary investigations suggest that once competent to cement, rock scallops retain this ability throughout their life. Size is an important biological factor affecting the cementing process. However, size of attachment varies among habitats, with some individuals never cementing. This indicates that factors in addition to size are required for permanent attachment. Evaluation of several physical characteristics of the attachment substrate found that substrate type, texture and contour influenced permanent attachment. Based on these findings, we have initiated pilot field studies to evaluate various grow-out techniques, and to assess the potential for manipulation of the cementing process of *C. gigantea*.

PEI MUSSEL AQUACULTURE: CHANGING TECHNOLOGIES. **T. Jeffrey Davidson,*** Atlantic Veterinary College, University of PEI, Charlottetown, PEI C1A due 4P3; **Richard K. Gallant,** Department of Fisheries and Tourism, Government of PEI, Charlottetown, PEI C1A 7N8.

Mussel aquaculture on PEI began in the late 1970's as an experiment, progressed to a pilot project and is now in full commercial production. At the onset of the industry, technology was not well refined. Many new ideas and innovations had to be developed to adapt to the PEI estuarine environment and especially to survive the harsh winter conditions. The industry's development and sustainability was particularly due to the innovation of the growers involved with the advancement of technologies. Because prices for mussels have remained stable to growers for the past 10 years, the industry has had to find innovative ways to decrease production costs and increase production. This presentation will outline some of the developments and advancements in technology that have taken place over the past 20 years in the culture, harvest, transport and processing of mussels. They include specialized aquaculture barges, hydraulic lifting equipment, innovative methods to decrease manual labour, improved culture equipment and techniques to control predators.

DESIGN AND EVALUATION OF FLOATING UPWELLER SYSTEMS FOR NURSERY CULTURE OF JUVENILE CLAMS AND OYSTERS. **Christopher V. Davis,*** Pemaquid Oyster Company, P.O. Box 302, Waldoboro, ME 04572; **Dale F. Leavitt,** Southeastern Massachusetts Aquaculture Center, c/o Hurley Library—Mass. Maritime Academy, 101 Academy Lane, Buzzards Bay, MA 02532; **Joseph A. Mariano,** Island Institute, 410 Main Street, Rockland, ME 04841.

Shellfish growers currently use various configurations of land-based and floating upwellers to expedite the nursery phase of commercial bivalve culture operations. Land-based upwellers systems typically allow for ease of maintenance and security, but depending on the elevation, tend to have high pumping costs. A benefit of Floating Upweller System (FLUPSY) designs is greatly reduced or zero pumping cost due to the minimal (2–10 cm) hydrostatic head required to force water through the upweller silos. Although raft-based tidal-powered systems have no power requirements, adequate flow rates are intermittent and limited to sites with suitable tidal flow. Water pump-based FLUPSYs typically employ centrifugal or axial flow electric pumps to propel water through the system. In contrast, low head airlift pumps are capable of moving large volumes of water, are inherently simple to construct and maintain, eliminate the risk of electrical shock, and have lower operating costs compared to most electrically driven water pumps. Pumping efficiency in airlift systems will be discussed as it relates to the air supply, depth of air injection, lift, diameter of the education pipe and air flow. This presentation will describe performance characteristics of FLUPSYs operated in New England waters and methods of optimizing the upweller design to both maximized growth rates and minimized operating costs.

THE USE OF A *SCHIZOCHYTRIUM* BASED DRY FEED FOR JUVENILE REARING AND BROODSTOCK CONDITIONING OF BIVALVE MOLLUSCS. **Jonathan P. Davis*** and **Clea R. Barenberg,** Taylor Resources Bivalve Hatchery, 701 Broad Spit Road, Quilcene, WA 98376.

Cultured live algae represent a significant cost associated with juvenile rearing of bivalves, including costs associated with brood stock conditioning of adults. The availability of a low cost, lipid-rich, heterotrophically grown *Schizochytrium* sp. based dry formulated feed (Sanders Brine Shrimp Company, Ogden, UT) has stimulated research on its use as a supplement for use in feeding larvae, post-set juveniles and adults in a variety of bivalves including mussels (*Mytilus galloprovincialis*) and geoduck clams (*Panopea abrupta*).

Feeding trials were conducted using the *Schizochytrium* based feed at three supplement levels in juvenile mussels and geoducks with differing results. Juvenile mussels maintained on the formulated feed at 33% and 67% and 100% supplemental levels, respectively demonstrated similar increases in growth compared to seed mussels grown on live algae only. Mussels fed at the 100% supplemental level, for example, grew at 3.2% per day compared to 3.9% per day for mussels fed live algae only. In addition, elevated levels of long chain fatty acids (primarily DHA) were observed in mussels fed the formulated feed compared to mussels fed live algae only.

Juvenile geoduck clams demonstrated the opposite result as clams maintained on the formulated diet at 50% and 75% supplemental levels showed reduced growth compared to clams maintained on live algae only. In both cases, juvenile mussels and geoducks were fed similar cell densities of live algae only, or combinations of live algae and disassociated *Schizochytrium* cells.

Additional feeding trials were conducted on adult brood stock geoducks at 25 and 50% supplemental levels. Feeding rates were maintained over a 4 week period and eggs from female clams assessed for fatty acid content and subsequent larval performance. These results, as well as the potential of utilizing dry formulated feeds are discussed in terms of the comparative costs of rearing live algae at commercial bivalve facilities.

APPLIED TECHNOLOGICAL DEVELOPMENT FOR HARD CLAM (*MERCENARIA MERCENARIA*) AQUACULTURE IN NEW JERSEY. George E. Flimlin, Jr., Rutgers Cooperative Extension, Toms River, NJ 08755.

The predominant form of shellfish culture in New Jersey coastal bays is that of the Hard Clam or Northern Quahog, *Mercenaria mercenaria.* Crop predation and biofouling of predator control screens are significant contributors to mortalities throughout the local industry. Inconsistent data on the exact numbers planted and later harvested also affect the true accounting of success.

Three small-scaled projects were initiated to address these problems. The first was the field testing of a high frequency sound blaster which was designed to repel blue crabs, *Callinectes sapidus,* from the planting area so that these crabs would not be entrained and thus covered over with the screen designed to exclude them during the planting operation. The second was the development of a device, which would clean the macroalgae from the surface of the predator control screens, so that the unchecked algal growth wouldn't suffocate the crop. Relying on a rotating brushes attached to a slightly buoyant submersible mechanical device, this machine may allow the grower to move away from the hand held scraper normally used. And the third, was the development of a seed counter that would allow the hatchery operator to better control the numbers of seed shipped or segregated for planting in individual plots.

FLAT OYSTER CULTURE IN NOVA SCOTIA: STRATEGIES TO OPTIMIZE THE GROWOUT OPERATION. A. L. Mallet* and **C. E. Carver,** 4 Columbo Drive Dartmouth, Nova Scotia, Canada, B2X 3H3.

This project was initiated to obtain strategic information on the effect of different rearing systems and protocols on the performance of European oysters from seed to market size. Several strategies were being practiced by the Nova Scotia oyster industry, but the lack of comparative data precluded an appropriate analysis of the various methods. For example, there were little information on the stocking densities for various oyster weight as well as the handling frequencies for various grow-out systems. Our strategy also need to take into account ice coverage during the winter months.

In this study, the final analysis does evaluate the biological performance of the oysters with the capital and operating costs for the various growout systems. Overall, 1-y-old oysters did better in lantern nets in terms of growth and survival. For 2-y-old oysters, similar performance is obtained in lantern nets and off-bottom tables whereas performance in 5-level suspended oyster bags was 30%–40% lower for the 1-y-old and 2-y-old oysters. Overall losses in tables were typically 40%, but certain operating conditions led to a loss of 70%. Fouling levels were minimal on oyster tables but was extensive on the suspended culture gear. Our calculations suggest a 1-fold difference in labor cost between handling suspended gear vs tables. The cost to produce an oyster, when handled frequently, suggests that the oyster table is the preferred method for final growout of flat oysters in Lunenburg.

CULTIVATION OF NATIVE OYSTERS AND NATIVE MUSSELS ON RAFTS, TRAYS AND ON THE BOTTOM IN MAINE, USA. Carter R. Newell,* Great Eastern Mussel Farms, Inc., P.O. Box 141, Tenants Harbor, ME 04860, Pemaquid Oyster Company, Damariscotta, ME, USA.

This talk will show current technology in mussel and oyster cultivation by two companies in Maine, USA. Oyster seed, after growing in a small hatchery and in upwellers, are transferred to floating trays where they grow to about 45 mm and are planted on the bottom at densities of 75 to 100 per square meter. The trademark name Pemaquid was developed early on and is held exclusively by Pemaquid Oyster Company for identification of its oysters in commerce. Rafts for overwintering small seed or holding 10,000 market-sized oysters are shown, utilizing trays from vinyl-coated wire for holding oysters of different sizes.

Mussels are grown on the bottom using techniques first developed in the Netherlands, and recent improvements in seed spreading will be discussed. New developments in mussel raft culture, using technology adapted from Scotland, Spain, the U.S. and Canada be demonstrated. Rafts of 30 ton capacity are seeded using a biodegradable cotton wrap and 12 meter, 1/2 inch polysteel ropes with polyethylene pegs and dropper weights. Final harvest yields of 5 to 10 pounds of mussel per foot of rope, higher than longline yields, is attributed to the use of pegs. Meat yields of over 50% have been achieved consistently in certain coastal Maine bays. Technology is currently being transferred among members of a mussel suspension culture working group of some 75 members.

A STUDY INVESTIGATING THE POTENTIAL OF AN ALTERNATIVE OYSTER SEED SOURCE FOR VIRGINIA AQUACULTURISTS. Francis X. O'Beirn* and **Mark W. Luckenbach,** Virginia Institute of Marine Science, Eastern Shore Laboratory, College of William and Mary, Wachapreague, VA 23480.

In Virginia, a consequence of the reduced harvest of wild oyster (*Crassostrea virginica*) stocks has been the development of intensive, hatchery-based oyster aquaculture. While this industry has

been growing steadily, one obstacle to its continued expansion is the lack of a consistent supply of seed oysters both in terms of quality and quantity. Aquaculturists have continually reiterated that the biggest impediment to their expansion is the paucity of seed oysters. East coast hatcheries are engaged in oyster seed production, but demand and disease exposure considerations limit availability. Consequently, we have investigated the feasibility of utilizing the slowest growing oysters produced from the hatchery (normally discarded) in a novel field nursery system. Four stocks were deployed in Spring 1998 and three stocks in Fall 1998, at two sites (Chincoteague and Wachapreague) on the Eastern Shore of Virginia. Controls were grown in similar conditions to the runt oysters. Growth and survival in each stock was monitored for 16 weeks. At the Chincoteague site, runt oysters performed equally as well as the controls. At the Wachapreague site, the controls outgrew the runt oysters. While there were some differences in the performances of the stocks within a site, major differences were apparent in growth and survival of stocks between the two growing areas. The utilization of such "runt" oysters may be feasible, if the aquaculturist is assured of good growing conditions. Otherwise the risks associated with the practice may not warrant the investment of time and resources.

ADVANCES IN THE CRYOPRESERVATION OF GAMETES AND LARVAE OF THE EASTERN OYSTER. Carmen G. Paniagua-Chavez,* John T. Buchanan, and **Terrence R. Tiersch,** Aquaculture Research Station, Louisiana Agricultural Experiment Station, Louisiana State University Agricultural Center, Baton Rouge, LA 70820; **John E. Supan,** Office of Sea Grant Development, Louisiana State University, Baton Rouge LA 70803.

Although the eastern oyster is considered to be an important species in the United States, little attention has been directed to the cryopreservation of gametes or larvae of this species. In this project, we developed techniques for cryopreservation of gametes and larvae, and we evaluated potential aquaculture applications to assist hatchery production. Preliminary studies of cryopreservation of oyster eggs were also performed. Dimethyl sulfoxide and propylene glycol plus sucrose were used to evaluate toxic effects of cryoprotectants. Eggs were cooled at 1.5 °C or 2.5 °C/min or plunged directly into liquid nitrogen. Dimethyl sulfoxide (0.88 M and 1.75 M) and sucrose (0.12 M, and 0.25 M) were the least toxic to eggs. The cooling rate yielding least damage to eggs was ñ1.5 °C/min, however, only an average of 14 eggs (out of 200) were stained with fluorescein diacetate and none were fertilizable. Sperm or trochophore larvae were frozen at ñ2.5 °C/min to ñ30 °C and plunged in liquid nitrogen. The optimum concentration of cryoprotectant was 10% propylene glycol for sperm and 10% or 15% propylene glycol for larvae. Frozen sperm and larvae were transported to an oyster hatchery at Grand Isle, Louisiana. Thawed sperm were used to fertilize fresh eggs. After 4 months, 1,000 oysters from the control group, 230 oysters produced from thawed sperm, 850 oysters from thawed larvae, and 57 oysters from natural spat fall were found. Oyster produced from thawed sperm and larvae developed normally in the hatchery, demonstrating opportunities for their use in research and in industry.

A LOW COST, FLOATING AXIAL-FLOW UPWELLER SHELLFISH NURSERY SYSTEM. Gregg Rivara,* Kim Tetrault, and **Michael Patricio,** Cornell Cooperative Extension, Suffolk County Marine Program, 3690 Cedar Beach Road, Southold, NY 11971.

Originally used in 1996 in Southold, New York the Cornell axial flow upweller has undergone some design changes making it easier to operate while cheaper to construct. The current design is based on a ten, fifty-five gallon silo module using a three-quarter horsepower "pump" that can be expanded easily.

During 1999, two million hard clams were grown to an average of 13 millimeters shell length in a four-module, forty-silo unit. The system will be discussed in terms of construction, stocking, and maintenance as well as building and operational costs. The pros and cons of such a system, especially compared to other systems, will also be discussed.

KUMAMATO OYSTER BROODSTOCK. Anja Robinson, Coastal Oregon Marine Experiment Station, Hatfield Marine Science Center, 2030 SE Marine Science Drive, Newport, OR 97366.

Kumamato oysters (*Crassostrea sikamea*) were brought to the west coast of the United States in 1947. Experimental seed was planted at various locations in Washington, Oregon and California. Plantings were successful and this encouraged several oyster growers to purchase commercial quantities of Kumamato seed oysters yearly. By 1953, a total of 3181 cases of seed were imported and grown for the cocktail oyster market. Once the hatchery technology was developed, Kumamato oyster seed was produced in hatcheries on the west coast of the United States.

In the early seventies, in the first commercial oyster hatchery in California, Kumamato and Pacific oysters were crossed in the hope of producing a large, deep-cupped oyster with good meat quality. However, the cross (called Gigamoto) grew up to be anything but what was hoped for. Since commercial oyster growers had mixed the cross with the rest of the Kumamato oyster seed, it was difficult to separate true Kumamato oysters from the cross. It was not until 1990 that genetic technologies became available to distinguish Kumamato oysters from Pacific oysters. Accordingly, the differences in the DNA pattern of true Kumamato oysters have been identified and commercial hatcheries have been provided with true Kumamato broodstock oysters so that they can produce pure Kumamato seed.

GROWTH OF JUVENILE CALICO SCALLOP, *ARGOPECTEN GIBBUS*, IN BERMUDA, AND ITS IMPLICATIONS FOR AQUACULTURE. **Samia Sarkis,* Doerte Horsfield, Greg Wells, Charles King,** and **Karen Smith,** Bermuda Biological Station for Research Inc., 17 Biological lane, Ferry Reach GEO1, Bermuda.

The calico scallop, *Argopecten gibbus*, has been studied as a candidate for aquaculture in Bermuda since 1996. Pediveligers (198.5 ± 16.0 μm) were set on day 11 after fertilisation on 3 mm black polyethylene netting in 1 micron filtered seawater at a density of 2.3 larvae · ml^{-1}: temperature was initially maintained at 22 °C ± 1 °C, and gradually decreased over a 12 day period to attain ambient (19 °C ± 1 °C). Post-larvae were given 18 cells · μl^{-1} on a daily basis; algal diet consisted of a mixture: *Tetraselmis chuii: Thalassiosira pseudonana* (clone 3H): *Isochrysis galbana* (clone: TISO)—. On April 8, 1998, day 12 after setting, cultch with settled spat was evenly distributed in 1 mm black polyethylene pouches and transferred to longlines in the field; mean shell height at time of transfer was <1 mm. Eight weeks later, spat were picked from cultch, measured and counted; mean shell height was 11.4 ± 1.4 mm. A subsample of 2000 spat was distributed in 3 mm Japanese pearl nets at an initial density of 150 spat · net^{-1}. Density was gradually reduced to 70 individuals · net^{-1} in July, and to 40 individuals · net^{-1} in September to allow for optimum growth. Monthly growth rate was monitored in terms of shell height, measured from a subsample of 30 scallops from three pearl nets; survival rate and sea surface temperature were also recorded monthly. Shell height increased to 48.0 ± 2.7 mm in 7 months; and was measured to be 58.3 ± 4.5 mm for 21 months old scallops. Growth rate ranged from 1.5 mm · $month^{-1}$ to 9.1 mm · $month^{-1}$; where maximum rates were associated with high sea surface temperatures (29.5 °C) during July and August. Survival rate was constant, recorded as >90% per net per month. With a potential local market value of $0.80 per animal, and a 7 months growth to market size following transfer to the field, the calico scallop is being further considered for aquaculture purposes in Bermuda.

TETRAPLOID EASTERN OYSTERS: AN ARDUOUS EFFORT. **John E. Supan,*** Office of Sea Grant Development, Louisiana State University, Baton Rouge, LA 70803; **Standish K. Allen, Jr.,** Virginia Institute of Marine Science, College of William & Mary, Gloucester Point, VA 23062; **Charles A. Wilson,** Coastal Fisheries Institute, Louisiana State University, Baton Rouge, LA 70803.

A surviving brood of tetraploid eastern oysters, *Crassostrea virginica* (Gmelin), was produced after eight attempts over four years, using three separate triploid broodstocks. The theory and technique were similar to those used for the production of tetraploid *C. gigas* (Thunberg). Ripe triploid female oysters, dubbed "blumoons" due to their rare occurrence, were sexually identified by a gonad smear and checked for ploidy by flow cytometry (FCM). Eggs were then stripped from the gonad, rinsed, and hydrated for 1 hour in filtered ambient seawater (FAS). After fertilization with haploid sperm, tetraploidy was induced by inhibiting the first polar body (PB1) with 0.5 mg/L cytochalasin B; the eggs were rinsed with 0.05% dimethyl sulfoxide in FAS when an untreated sub-sample of eggs exhibited 50% PB1. Larvae and resulting spat were reared using standard techniques. Earlier attempts were stymied by the rarity of blumoons in the triploid broodstock, or poor larval survival. In the three latest attempts, 1,600 putative triploids were opened to verify sex and ploidy. Between 0 and 10 blumoons were identified for tetraploid attempts in summers of 1997 and 1998. In June, 1999, the attempt was successful because older triploids increased the female:male ratio and provided more blumoons for tetraploid spawns. Approximately 500 juvenile oysters survived from five larval broods of which 42% were tetraploid. The seed are being maintained at the Sea Grant Oyster Hatchery in Grand Isle, Louisiana and at the Virginia Institute of Marine Science Aquaculture Genetics and Breeding Technology Center. Preliminary FCM analyses of some individual oysters indicate the occurrence of 2n/3n/4n mosaicism and 2n/4n individuals, probably due to 2n sperm. During indifferent or early gametogenic development (Jan.–Feb., 2000), individual tetraploid adults will be identified by FCM prior to conducting 4n × 4n and 4n × 2n crosses to confirm the resulting production of tetraploid and triploid generations, respectively.

WATER QUALITY AND HARMFUL BACTERIA

USE OF HIGH HYDROSTATIC PRESSURE TO CONTROL PATHOGENS IN RAW OYSTERS. **Haejung An,* Hakan Calik, Haian He, Roger Adams,** and **Michael T. Morrissey,** Oregon State University-Seafood Lab, Astoria, OR 97103.

Raw oysters are the main vehicle of transmitting diseases induced by *Vibrio* spp. In 1997, over 80 confirmed cases of *Vibrio parahaemolyticus* (Vp) were reported in the Pacific Northwest, and the cases has lingered in the last two years although the numbers diminished. The naturally occurring *Vibrio* spp. in oysters can be controlled by use of a new technology, high hydrostatic pressure (HHP) treatment. HHP is a nonthermal process which destroys microorganisms with a minimal effect on overall appearance, taste and texture of raw foods.

We are determining the effects of HHP treatment on Vp in oysters to establish optimum HHP conditions for processing time and pressure and evaluate the sensory changes related to HHP treatment during storage. Different strains of Vp isolated from the

commercial oysters and the clinically confirmed virulent Vp strains obtained from FDA, Seattle, WA were tested for HHP effects at different settings of pressure (35–50K psi) and time (10 sec–15 min). The results showed that both environmental and clinical Vp strains were equally susceptible to HHP treatment. The optimum conditions for reducing pure culture of Vp from 10^9 cfu/ml to 10^1 cfu/ml was achieved at 50K psi in 30 sec, while it took 14.5 min at 35K psi. On the processing setting suitable for commercial oysters, generally a 2–3 log reduction of total microbial counts was observed. Sensory test showed HHP treated oysters received the higher quality scores than the control group during storage up to 22 days, demonstrating that HHP is a satisfactory means to control pathogens in raw oysters.

CAN THE TILLAMOOK COUNTY PERFORMANCE PARTNERSHIP RESTORE THE ECOLOGICAL BALANCE OF TILLAMOOK BAY? **Deborah Cannon**, Shellfish Program Specialist, Food Safety Division, Oregon Department of Agriculture, 635 Capitol St. NE, Salem, OR 97301; **Kim Hatfield**, Scientific/Technical Program Coordinator, Tillamook County Performance Partnership/Tillamook Bay National Estuary Project, 613 Commercial/PO Box 493, Garibaldi, OR 97118.

In 1999 the Tillamook Bay National Estuary Project completed its Comprehensive Conservation and Management Plan for Tillamook Bay, Oregon. The TBNEP, funded by the US Environmental Protection Agency's National Estuary Program identified four priority problems one of which is bacterial contamination and other water quality issues, its effects on shellfish harvesting and other human uses. The Tillamook County Performance Partnership, which succeeds the TBNEP, has committed to implementing sixty-three action plans from the CCMP by 2010. This paper discusses the challenges to meeting the stated goals: achieve water quality standards for bacteria in rivers and Bay by 2010; document at least a 25% reduction in bacteria loads to rivers, with apparent trends by 2005 and statistically significant results by 2010; and achieve at least a 25% reduction every four years in the number of days that rivers are not in compliance with water quality standards for bacteria.

THE VARIOUS RELATIONSHIPS BETWEEN SHELLFISH AND WATER QUALITY. **William F. Dewey**, Taylor Shellfish Company, Inc., 130 SE Lynch Road, Shelton, WA 98584.

Shellfish Growers dedicate considerable resources to protect and restore clean water to produce wholesome shellfish which are safe to consume raw. This is the foundation of the National Shellfish Sanitation Program. Recognized as equally important is the role that filter feeding molluscan shellfish play in cleansing water. Touted as keystone species, significant efforts have been made to reintroduce oysters to New York Harbor and the Chesapeake Bay to improve water quality in these systems. Aside from the valuable function the oysters physically serve as habitat and refuge is their ability to filter huge volumes of phytoplankton which, in heavy blooms, can cause low dissolved oxygen problems when the blooms die off as well as blocking critical sunlight for seagrasses and macroalgae. In the Pacific Northwest a citizens group, organized in opposition to mussel farm expansion on the basis of the aesthetic impact, is attempting to change the water-cleansing image of shellfish into a water-polluting image. The group recently filed suit in federal court claiming the feces, pseudofeces, mussel shell debris and escaped mussel spat from propagating mussels are a point source of pollution and require a National Pollution Discharge Elimination System (NPDES) permit under the Clean Water Act. This paper discusses the dichotomy between the views of shellfish as polluters versus the view of shellfish as capable of improving water quality and habitat.

SHELLFISH WATER QUALITY TRENDS AND THREATS IN PUGET SOUND. **Stuart D. Glasoe*** and **Duane Fagergren**, Office of the Governor, Puget Sound Water Quality Action Team, P.O. Box 40900, Olympia, WA 98504-0900.

Puget Sound has some of the world's finest habitat for the cultivation of clams, mussels and oysters. Commercially, these products yield an annual farm-gate value of nearly $50 million. In broader terms, shellfish harvesting is a cherished part of Puget Sound's rich heritage and quality of life, and serves as a key measure of the estuary's environmental health.

In the 1980s a number of the Sound's commercial shellfish areas were downgraded primarily because of nonpoint source pollution and additional monitoring information. This decline stabilized in the 1990s as a result of targeted efforts to restore water quality. A great success story, right? A broader review presents a mixed picture and forecasts an uncertain future for the Sound's shellfish tidelands, especially given the region's fast-growing population. Upgrades over the past decade have generally been offset by an equal number of downgrades. Some successful restorations have been reversed by recurring problems. Other sites have never recovered. And the harvesting classifications in most restored areas are tenuous, requiring constant monitoring and follow-up work.

Given the persistent nature of these water quality threats, are we using our tools and resources to achieve temporary fixes or to make lasting changes? Do we have the vision and resolve to manage growth and control pollution in ways that will effectively preserve our environmentally sensitive tidelands, or are we carrying out a mission of haphazard restorations? Experiences in such areas as Drayton Harbor, Burley Lagoon and Lower Hood Canal provide some insight to these questions.

THE USE OF MULTIPLE ANTIBIOTIC RESISTANCE AND MOLECULAR TECHNIQUES (PULSED FIELD GEL ELECTROPHORESIS AND RIBOTYPING) FOR IDENTIFYING COLIFORM POLLUTION SOURCES. **G. I. Scott,* M. H. Fulton, B. C. Thompson, L. F. Webster, A. K. Leight, E. F. Wirth,** and **J. Stewart,** NOAA/NOS, CCEHBR, Charleston, SC; **G. P. Richards,** Dept. of Agriculture, Delaware State Univ., Dover, DE; **D. Chestnut,** SC Dept. of Health and Env. Control, Columbia, SC; **R. F. Van Dolah,** SC Dept. of Natural Resources, Charleston, SC; **S. Parveen,** Univ. of Florida, Gainesville, FL.

Urbanization may result in significant inputs of contaminants into salt marsh ecosystems. Significant discharges of bacterial pollution from septic tanks and combined sewer overflows may occur, often resulting in closure of shellfish harvesting waters due to the presence of bacterial pollution. In addition, wildlife coliform pollution sources may be present and contribute to shellfish closures. There is a clear need to develop methods for differentiating human versus wildlife coliform bacterial sources. Several novel methods for differentiating human and wildlife coliform bacterial sources were evaluated including Multiple Antibiotic Resistance (MAR), Pulsed Field Gel Electrophoresis (PFGE), Ribotyping (RT) and Fatty Acid Profiling (FAP). Surface water samples were collected from several estuaries in SC as well as selected sewage treatment plants and septic tanks. All samples were enumerated for fecal coliform bacterial densities (MPNs). Samples were then analyzed by API biotyping to isolate *E. coli* and were further analyzed by MAR, PFGE, and RT. Results indicated that the % of *E. coli* comprising the coliform group was increased with increasing urbanization and high MAR was found in areas adjoining sites with septic tanks or influenced by sewer discharges. Wildlife areas had negative MARs and a much lower % of *E. coli*. PFGE and RT provided DNA differentiation of bacterial pollution sources including septic tanks and domestic animal sources. These findings indicate that these methods may be helpful in identifying different sources of fecal coliform bacteria.

DISTRIBUTION OF *VIBRIO PARAHAEMOLYTICUS* IN PUGET SOUND OYSTERS, WATER, AND SEDIMENTS DURING SUMMER 1999. **Russell P. Herwig*** and **Robyn M. Estes,** School of Fisheries, University of Washington, 3707 Brooklyn Ave NE, Seattle, WA 98105; **Cindy L. Messey** and **Daniel P. Cheney,** Pacific Shellfish Institute, 120 State Ave NE, #142, Olympia, WA 98501.

Vibrio parahaemolyticus (*Vp*) is a bacterium that is naturally found in estuarine and marine waters around the world. This organism can accumulate in filter-feeding organisms, such as oysters, and be a problem with shellfish that are harvested during the summer months. Selected strains of *Vp* may cause food borne illness. In 1997 and 1998, several outbreaks of *Vp* gastroenteritis were caused by the consumption of raw oysters harvested from the Pacific Northwest. During the summer of 1999, oyster, sediment, and water samples were collected from four sites in Puget Sound, Washington. Levels of *Vp* were determined using a newly developed molecular Most Probable Number (MPN) method. Samples were blended, diluted, and inoculated into a series of tubes containing Alkaline Peptone Water (APW). Small aliquots of liquid were removed from each incubated tube and used in a polymerase chain reaction (PCR). This molecular method amplified a specific fragment of DNA found in *Vp*. Using the new method, the enumeration of *Vp* was performed within 2 days, a much shorter time compared to the FDA-approved method. Our results indicated that levels of *Vp* in Puget Sound oysters, sediment, and water were not detectable or very low in June 1999, increased at selected sites in July, and remained elevated in August. Concentrations of *Vp* were much higher in sediment compared to water samples. Although the different Puget Sound sites had comparable water temperatures and salinities during the summer, a site on Hood Canal had much higher *Vp* levels in samples collected during July and August. The reasons for the elevated levels of *Vp* at particular locations in Puget Sound are not understood.

OUTBREAKS OF *VIBRIO PARAHAEMOLYTICUS* GASTROENTERITIS FROM RAW OYSTER CONSUMPTION: ASSESSING THE RISK OF CONSUMPTION AND GENETIC METHODS FOR DETECTION OF PATHOGENIC STRAINS. **Charles A. Kaysner*** and **Angelo DePaola, Jr.,** Food and Drug Administration, Bothell, WA 98021 and Dauphin Island, AL 36528.

During the summers of 1997 and 1998, large outbreaks of *V. parahaemolyticus* gastroenteritis occurred from the consumption of raw oysters in the US. The West Coast outbreak was the first to have occurred in this country from the consumption of raw molluscan shellfish; over 200 culture-confirmed cases were identified. Over 400 cases were confirmed from oysters harvested from Galveston Bay in Texas and 20 cases were confirmed in New York and Connecticut from oysters originating from Long Island Sound. Distinct serogroups of the pathogen were responsible for illnesses on the West Coast in contrast to those on the East and Gulf Coasts. Monitoring of shellfish samples by State and federal authorities found low levels of *V. parahaemolyticus* in all implicated growing areas, suggesting strains of low infectious dose. FDA has completed a risk assessment study for consumption of raw molluscan shellfish as part of the Food Safety Initiative. Newly developed genetic techniques were employed for the first time to determine levels of *V. parahaemolyticus* in shellfish and detect the pathogenic strains of the species.

DOES COMMUNITY INVOLVEMENT LEAD TO LONG-TERM POLLUTION SOLUTIONS? T. L. King, Washington Sea Grant Program, 11840 North Hwy 101, Shelton, WA 98584.

In Puget Sound, innovative education programs have helped community volunteers to reduce pollution sources to the point of reopening shellfish growing areas. Septic soldiers, fecal ferrets, septic socials, and cranberry PIE programs have all helped to educate and influence changes, but is this enough? Will it last? Using case studies from Hood Canal and southern Puget Sound communities, we will look at the impact these programs have had two and three years later.

ISSC'S RESEARCH INITIATIVES. Ken B. Moore, Executive Director, Interstate Shellfish Sanitation Conference 115 Atrium Way, Suite 117, Columbia, SC 29223.

The Interstate Shellfish Sanitation Conference (ISSC) was organized in 1982 to address the safety and sanitation of molluscan shellfish (oysters, clams, and whole and roe-on scallops). The ISSC has a formal Memorandum of Understanding with the U.S. Food and Drug Administration (FDA) to promote shellfish sanitation through the National Shellfish Sanitation Program (NSSP), a tripartite cooperative program of Federal and State public health officials and the shellfish industry working together to improve shellfish safety. The ISSC recognizes that the NSSP is founded on the premise that, through appropriate controls, molluscan shellfish can be consumed raw by most people without reasonable risk. The ISSC remains committed to that promise. In 1994, the ISSC established an Executive Office and began to expand the activities of the organization to more effectively coordinate and facilitate implementation of the NSSP. The office acquired funding from several federal agencies. A significant amount of these funds have been directed to address research needs associated with the NSSP implementations. To date approximately $400,000 has been awarded to researchers to address issues associated with molluscan shellfish public health. The presentation will focus on the organization, its research goals and its research activities.

ELIMINATION OF *VIBRIO* CONTAMINATION IN RAW IN-SHELL OYSTERS THROUGH LOW TEMPERATURE PASTEURIZATION. D. L. Park,* Department of Food Science, Louisiana Agricultural Experiment Station, Louisiana State University, Baton Rouge, LA 70803; **L. S. Andrews,** Sugar Processing Research Institute 1100 Robert E. Lee Blvd., New Orleans, LA 70124; **Y-P Chen,** Department of Human Ecology, Tainan College of Art and Technology, 1-2 52, Sheng-Li Rd., Tainan, Taiwan, 701.

Vibrio vulnificus and *V. parahaemolyticus* are natural inhabitants of estuarine environments and may be transmitted to humans by ingestion of raw oysters. This study focused on the use of low temperature pasteurization, to reduce these *Vibrio* species to non-detectable levels thus reducing the risk of infection associated with raw oyster consumption. In-shell oysters artificially inoculated with *V. vulnificus* and *V. parahaemolyticus* and live oysters naturally contaminated with *V. vulnificus* were heat treated with low temperature pasteurization of 50 °C for up to 15 minutes. Samples of processed and unprocessed oysters were enumerated for *V. vulnificus, V. parahaemolyticus,* and aerobic spoilage bacteria during a 0–14 day period. Low temperature pasteurization was effective in reducing these pathogens from >100,000 to nondetectable levels in less than 10 minutes of processing. Spoilage bacteria were reduced by 2–3 logs increasing the shelf-life in excess of 7 days beyond live unprocessed oysters.

POSTERS

FREQUENCY OF SHELL REPAIRS IN COMMON CLAMS FROM NEW JERSEY. Richard R. Alexander,* Dept. Of Geological & Marine Science, Rider University, Lawrenceville, NJ 08648; **Gregory Dietl,** Dept. of Zoology, North Carolina State University, Raleigh, NC 27695.

Shells of *Argopecten irradians, Anadara ovalis, Anomia simplex, Divaricella quadrisulacta, Donax variabilis, Ensis directus, Spisula solidissima,* and *Tagelus plebeius* were collected from the sandy tidal flat near Hereford Inlet, NJ (n = 5102). The salt marsh and adjoining muddy tidal flat at Tuckerton NJ provided shells of *Crassostrea virginica, Geukensia demissa, Mercenaria mercenaria, Mya arenaria, Mytilus edulis,* and *Petricola pholadiformis* (n = 4411). *Astarte castanea* was sampled from the beaches at Belmar, NJ (n = 1000). Size-frequency distributions for shell lengths (widths) at death versus lengths (widths) at inception of shell repair reveal repair frequency (% of sample) and any size refuge from sublethal breakage. Repair position was coded as dorsal, ventral, anterior, or posterior. Geometry of repaired breakage was coded as scalloped, divoted, cleft, or embayed. *D. quadrisulcata* lacks shell repairs. Repairs in shells of *D. variabilis* (<<1% of sample) and *A. castanea* (1%) are usually localized ventrally as scalloped margins. *A. irradians* bear mostly clefts (<2%) between radial ribs. Repaired valves in *P. pholadiformis* (3%) show posteriorly concentrated scalloped margins. Repaired shells of *S. solidissima* (6%) include many embayed fractures on the ventral-posterior. A size refuge from sublethal fracture exists. Shell repairs in *T. plebeius* (5%) are located anteriorly, ventrally, and posteriorly. Repairs in *A. ovalis* (8%) are concentrated ventrally, where the foot protrudes. Shell repairs in *G. demissa* (10%) are posteriorly concentrated divots mostly in smaller size-classes. All types of shell repairs occur around the commissure of *C. virginica* (11%), but most are anterior scalloped margins. Shell repairs in *M. mercenaria* (12%) are mostly posterior triangular divots that occur over a wide range of size classes and are usually proximal to where siphons protruded. For *M. edulis,* shell repairs (19%) of all four

types occur at all shell widths, but are located posteriorly almost exclusively. Repairs in *A. simplex* (22%) occur at all sizes and are concentrated ventrally. Shell repairs in *E. directus* (21%) occur at all size classes as posteriorly scalloped margins near where siphons are incompletely withdrawn. Repairs in *M. arenaria* (31%) occur over a wide range of shell widths at the anterior, posterior, and ventral margins where the foot, siphons, and mantle, respectively, are exposed when valves are closed.

SELECTION AND GROWTH OF *CRASSOSTREA VIRGINICA* BASED ON WATER QUALITY. Troy D. Alphin* and **Martin H. Posey**, Depart. Biological Sciences, University of North Carolina at Wilmington, Wilmington, NC 28403; **David W. Freshwater** and **Robert A. York,** Center for Marine Science Research, Wilmington, NC 28403.

The eastern oyster, *Crassostrea virginica,* has experienced population declines over the last few decades throughout the Atlantic and Gulf coasts, causing concern among both fishermen and resource managers. This decline has been attributed to overharvest, disease, and declining water quality and is currently the focus of many studies. In North Carolina, as in other states, programs are in place to help reverse this trend, through oyster relay programs, placement of oyster shell to promote settlement, and experimental breeding programs. Experimental breeding and outplant programs have had mixed success, especially when attempting to establish populations in impacted systems. One contributing factor has been suggested to be differences between populations having long-term exposure to eutrophic and/or turbid conditions. This would suggest the need for using differing parental stocks in certain outplant programs. This project focuses on assessing the feasibility of using Inter-SSR and RAPD methods for detecting possible population structure and water quality specific markers in oysters. In addition to genetic techniques, size distribution and density data will be collected from the same oyster populations and correlated with background water quality.

GAMMA IRRADIATION EFFECTS ON EARLY LIFE STAGES OF THE EASTERN OYSTER. Ingrid Ardjosoediro,* Nyanti Lee, John Supan, and **Terrence R. Tiersch,** Aquaculture Research Station, Louisiana Agricultural Experiment Station, Louisiana State University Agricultural Center, Baton Rouge, LA 70820; **John E. Supan,** Office of Sea Grant Development, Louisiana State University, Baton Rouge, LA 70803.

The application of radiation has been used in invertebrate studies to investigate growth increases, and to induce sterility, such as the control of agricultural pest species. The possibility of creating a sterile oyster is of importance for the production of higher meat yields during the spawning season and for the use of transgenic oysters and exotic species. Preliminary studies were performed during May to July of 1998 to evaluate the effects of gamma irradiation on larvae of the eastern oyster. Larvae were irradiated at 24 h or 48 h after fertilization, using a cobalt-60 gamma irradiator (Shepherd model) at doses of 0, 3, 5, 10, 15, 20, or 25 krad, at a rate of 1528 rad/min. There were significant differences in mortality between 0 and 5 krad and between 3 and 5 krad in the 48-h larvae at 24 h after irradiation. At 36 h after irradiation, these larvae showed no significant differences in mortality among treatments. These preliminary results indicate that the effects of gamma radiation are dependent on larval stage and time after irradiation.

This project was continued during the summer of 1999 when the larval stages trochophore, D-stage, and umbo were exposed to gamma radiation at doses of 0, 5, 10, 15, or 20 krad. Growth and survival was monitored at 2 d, 4 d, and 6 d after radiation. It was observed that resistance to radiation increased with the developmental stage of the larvae. These studies provide an initial step in evaluation of the utility of irradiation in oyster production. Further studies will address the reproductive ability of irradiated oysters.

FEEDING SELECTIVITY OF NATIVE FRESHWATER MUSSELS (UNIONIDAE) AND COMPETITION WITH ZEBRA MUSSELS. Shirley M. Baker* and **Jeffrey S. Levinton,** Dept. Ecology and Evolution, State University of New York, Stony Brook, 11790.

The invasion of the Hudson River by the zebra mussel (*Dreissena polymorpha*) has resulted in a decline of cyanobacteria, *Microcystis* in particular, and a rise to dominance by diatoms. Since the invasion, the density of native mussels (Family Unionidae) has declined 30–90%. Our objective was to examine feeding selectivity in unionid mussels and determine the potential competition for food between native mussels and zebra mussels. We examined feeding rate and feeding selectivity of several species of native mussels, using flow cytometry. We found that, like zebra mussels, unionids are capable of efficiently sorting and rejecting particles. As in zebra mussels, the cyanobacterium *Microcystis* was preferentially ingested by unionids over almost all other particle types tested. One exception, however, was the unionid *Amblema plicata,* which preferentially ingested a diatom, *Cyclotella,* over *Microcystis. Amblema* has been less severely affected by zebra mussels than have many other unionid species. Like zebra mussels, unionids vary their clearance rates according to the composition of the suspension. Unionid clearance rates were significantly lower in the presence of typically rejected particles such as the large green alga, *Scenedesmus.* Although *Microcystis* was a preferred particle type, unionid clearance rates were not stimulated by its presence to the same degree as zebra mussel clearance rates are. Our selectivity and clearance rate data suggest that varying degrees of competition with zebra mussels for particular, and limited, food types may be an underlying factor in the decline of native mussel abundance and diversity.

VIABLE BUT NON-CULTURABLE RESPONSE FOR PHASE VARIANTS OF *VIBRIO VULNIFICUS* IN CLAMS. **Wafa Birbari, Anita Wright,*** and **Gary Rodrick,** Food Science and Human Nutrition Dept., University of Florida, Gainesville, FL 32611.

Vibrio vulnificus is the leading cause of human mortalities from bacterial infections associated with the consumption of seafood, particularly raw oysters. Decreased disease prevalence correlates with colder water temperatures that have been shown to induce a viable but non-culturable (VBNC) state. Resuscitation to the culturable state is achieved by elevating incubation temperature prior to cultivation. *V. vulnificus* also exhibits phase variation between opaque (O) and translucent (T) colony morphologies that correlates with capsule expression. In microcosms, both phase variants were shown to exhibit similar kinetics for induction and resuscitation of VBNC cells, and morphotypes remained stable. VBNC cells could be resuscitated from clams after 48 h. Uptake of *V. vulnificus* in clams induced a conversion of O to T morphotype but not in the other direction. Studies are ongoing using mutants with genetically defined disruptions of the CPS locus.

PERKINSUS MARINUS* POPULATION DYNAMICS IN NORTH INLET, SOUTH CAROLINA—AN ECOSYSTEM MODEL.** **Jodi Brewster and **Dave Bushek,** Baruch Marine Field Laboratory, Baruch Institute for Marine Biology and Coastal Research, Georgetown, SC 29442; **Richard Dame,** Department of Marine Science, Coastal Carolina University, Conway, SC 29528.

Perkinsus marinus, a protozoan parasite of the eastern oyster (*Crassostrea virginica*), influences oyster population dynamics in estuarine ecosystems on the east and Gulf coasts of the United States. It is the causative agent of "dermo" disease that has contributed to the collapse of eastern oyster fisheries. The parasite can, however, exist within an estuary without causing extensive mortalities. The mechanisms that regulate *P. marinus* populations at the ecosystem level, and thus its impact on oyster populations, remain unclear. The parasite is transmitted through the water column, encountering host oysters as they filter the water. Oysters can remove a portion of the filtered parasites, but many will invade tissues and proliferate within the oysters. Those factors that determine the fate of parasites within the oysters and during planktonic transmission are key to understanding the mechanisms that affect *P. marinus* epizootics within the oyster population. A box model was constructed using Stella 5.1 software to simulate the populations dynamics of *P. marinus* in North Inlet Estuary, South Carolina. Our objective is to develop a model that can be used to test new concepts and hypotheses, and to evaluate consequences of climate change, estuarine variability, and anthropogenic impacts on the *P. marinus* populations. The information obtained should help define better oyster reef management strategies.

PERKINSUS DISEASE PROGRESSION IN FIELD OYSTERS: A MODELING STUDY. **Diane J. Brousseau,** Fairfield University, Fairfield, CT 06430; **Jenny A. Baglivo,** Boston College, Chestnut Hill, MA 02467.

During 1997, oysters from six locations in Connecticut (Black Rock Harbor, Bridgeport; Saugatuck River, Westport; Thames River, Waterford; Mystic River, Stonington), New York (Oyster Bay) and Massachusetts (Cotuit) were analyzed for the presence of *Perkinsus marinus* and water temperatures were monitored at each site. Median values of disease prevalence were between 96% and 100% for adult oysters and between 30% and 68% for juveniles. Infection levels in oyster parasites began climbing when water temperatures reached 13–16 °C at the four Connecticut sites. These results substantiate earlier observations made for the Bridgeport site and suggest a different pattern of infection development from that reported in oysters from locations further south, where temperatures $\geq$20 °C are required. A temperature-disease course model was developed to predict the effect of seasonal water temperature changes on disease progression of *P. marinus* in field populations of *Crassostrea virginica* in Long Island Sound. This model allows the grower/manager to predict Dermo intensity in shellfish beds if field water temperature patterns are known. Such information can be used to select oyster growout beds and determine optimal time to harvest.

IDENTIFICATION OF A SERINE PROTEASE GENE IN *PERKINSUS MARINUS*. **Gwynne D. Brown*** and **Kimberly S. Reece,** Virginia Institute of Marine Science, The College of William and Mary, Gloucester Point, VA 23062.

Perkinsus marinus was identified as the causative agent of Dermo disease in the eastern oyster, *Crassostrea virginica,* in the late 1940's. Fifty years later *P. marinus* has expanded its geographic range, heavily impacting previously unaffected oyster populations of the Northeast, yet little is known regarding the pathogenic mechanisms of this parasite. Identification of proteins whose activities and gene expression levels correlate with virulence will promote an understanding of disease mechanisms and facilitate the development of more effective disease management strategies. Proteases have been found to play a key role in pathogenesis of several parasitic protozoans. We have identified a serine protease gene from *P. marinus* using "universal" degenerate primers in the polymerase chain reaction to amplify a 475 bp fragment. This gene appears to be closely related to the subtilisin gene family of serine proteases. Using digoxigenin to label the amplified fragment we have screened a *P. marinus* λ phage genomic library. DNA from hybridizing phage has been isolated and subjected to Southern blot analysis. At least two different types of recombinant clones have been identified. We are currently in the process of subcloning and sequencing the DNA fragments to characterize the complete serine protease genes.

RESEARCH-SCALE CULTURE OF OYSTER LARVAE. **John T. Buchanan,*** Department of Oceanography and Coastal Sciences, Louisiana State University, Baton Rouge, LA 70803; **Carmen G. Paniagua** and **Terrence R. Tiersch,** Aquaculture Research Station, Louisiana State University Agricultural Center, Baton Rouge, LA 70820; **Richard K. Cooper,** Department of Veterinary Science, Louisiana State University, Baton Rouge, LA 70803.

The eastern oyster, *Crassostrea virginica,* comprises an important national fishery. Research on gene transfer and cryopreservation of gametes and larvae can lead to improvement in this industry, although it was necessary to develop research-scale techniques for the holding of oysters and the culture of larvae. Along with consistent production of high quality gametes and larvae, consideration must be given to experimental replication, avoidance of contamination, and containment of genetically-modified organisms. All of our work was done with artificial seawater in recirculating systems over 100 km from the nearest coastal area. We examined the effect of several variables on the production of gametes and larvae of the eastern oyster. First, we developed protocols for holding broodstock in the laboratory, acclimation to laboratory conditions, and collection of gametes. We developed methods to assay gamete quality and for cold storage of gametes. Second, we optimized methods for the small-scale production of oyster larvae by artificial fertilization by examining the effect of container volume, aeration, and artificial water source on larval survival. Using these techniques, we successfully transferred the gene for red-shifted green fluorescent protein (*rsGFP*) into oyster sperm, embryos and adults. These techniques have also been used in experiments to cryopreserve eastern oyster sperm and trochophore larvae. A program such as this would be useful for the small-scale production and culture of shellfish larvae for a variety of experimental purposes.

THE EFFECT OF OYSTER REMOVAL ON INTENSITIES OF *PERKINSUS MARINUS* INFECTIONS IN NATIVE OYSTER POPULATIONS. **Emily Butsic*** and **Richard Dame,** Department of Marine Science, Coastal Carolina University, Conway, SC 29526; **David Bushek,** Baruch Marine Field Laboratory, University of South Carolina, Georgetown, SC 29442.

The parasitic oyster pathogen *Perkinsus marinus* has caused many problems for the east and Gulf Coast oyster industries. Recommended management strategies include fallowing beds after removing infected oysters and timing the planting and harvesting of oysters around seasonal infection cycles. To examine the effectiveness of the fallowing strategy, we measured *P. marinus* infection levels before and after the removal of native oysters. Oysters from three to five locations along each of eight intertidal creeks in North Inlet Estuary, South Carolina were processed by Ray's fluid thioglycollate medium tissue assay for *P. marinus.* Infection intensities were similar among all eight creeks in July of 1997. During January–February 1998, oysters were removed from four of the eight creeks and subsequently allowed to repopulate via natural recruitment. In early August 1999, the creeks from which the oysters had been removed showed lower levels of *P. marinus* than control creeks. These observations appear to indicate that fallowing oyster beds may help control *P. marinus* infections and minimize subsequent oyster mortality. Other studies, however, have shown that *P. marinus*-free oysters placed in these same eight creeks contracted infections at the same rates. The lower intensities observed in August 1999, in the creeks where oysters had been removed, was an artifact of age. Oysters in these creeks were younger and therefore had less time to develop infections than the older oysters in the control creeks. Thus, early harvesting may be may still be required to avoid mortalities by *P. marinus.*

DEVELOPMENT AND VERIFICATION OF A SIMPLE MODEL FOR *PERKINSUS MARINUS* ABUNDANCE IN CHESAPEAKE BAY OYSTERS. **Lisa M. Ragone Calvo*** and **Eugene M. Burreson,** Virginia Institute of Marine Science, College of William and Mary, Gloucester Point, VA 23062.

A simulation model was developed to investigate the population dynamics of the protistan parasite, *Perkinsus marinus,* within its host, the eastern oyster, *Crassostrea virginica.* The main objective was to evaluate the relationship between *P. marinus* population dynamics and environmental conditions in order to predict the onset and termination of *P. marinus* epizootics in Chesapeake Bay oyster populations. The model was calibrated using data derived from laboratory experiments and from field observations of *P. marinus* prevalence and intensity in the James River, Virginia for the years 1990–1993. The relatively simple, individual based model is driven by temperature and salinity and tracks *in vivo* parasite density through time at a daily time step. The model was verified with five years (1994–1998) of monthly field observations of parasite abundance at three oyster bars located along a salinity gradient in the James River. Five year simulations, initiated on Julian day 1 with a single parasite input corresponding to the actual observed abundance for that month, significantly correlated ($p < 0.001$, $r^2 = 0.439$ to 0.729) with observed abundances of *P. marinus* at the three James River oyster bars. Predicted parasite abundances were stabilized during the 5 year simulation for all three oyster populations without the additional input of transmission events, suggesting that a single transmission event can result in *P. marinus* becoming enzootic in an area for a long period of time.

POTENTIAL TRIPLOID PRODUCTION OF OYSTERS USING SECOND METAPHASE OOCYTES. **Gregory M. Coates,*** Aquaculture Research Station, Louisiana State University Agricultural Center, Louisiana Agricultural Experiment Station, Baton Rouge, LA 70820; **John E. Supan,** Office of Sea Grant Development, Louisiana State University, Baton Rouge, LA 70803.

Due to the fact that tetraploid eastern oysters *Crassostrea virginica* are not yet available to spawn with diploids to produce all-triploid populations (interploid triploids), successful triploid production of *C. virginica* is dependent on chemical induction usually with cytochalasin B (CB). Treatment efficiency with CB usually varies significantly due to asynchronous meiotic maturation of the oocytes dissected from ripe adult oysters. The neurohormone serotonin (5-hydroxytriptamine, 5-HT) and alkaline seawater (prepared with 0.1 M NaOH), used previously on other molluscan species to re-initiate meiosis, were used separately to treat unfertilized oocytes prior to fertilization. Dissected oocytes that normally arrest at either the first prophase (germinal vesicle, GV) or first metaphase (germinal vesicle breakdown, GVBD) of meiosis, prior to fertilization, were exposed to 5-HT and alkaline seawater to examine the effect on oocyte development.

Oocytes from ripe adult oysters were removed, rinsed, and exposed to various 5-HT concentrations (1 μM, 10 μM, and 100 μM) and four different pH concentrations (7.0, 8.0, 9.3, 10.0) in filtered seawater at 25 °C. Exposure to 5-HT caused a significant increase ($P < 0.05$) in meiotic maturation rate when compared to the control. Oocytes matured to second metaphase (first polar body stage) in all 5-HT treatments and the control. Significant increases ($P < 0.05$) in the rate and stage of meiotic maturation occurred in the alkaline treatments (pH 9.3 and 10.0). Alkaline seawater activated the oocytes not only to first polar body stage, but also to second polar body stage and cleavage. Oocytes that reached first polar body stage could be fertilized and treated with CB immediately after fertilization to inhibit the second polar body and produce triploids. Using activated oocytes may improve triploid induction techniques by reducing variation in the stage of meiotic development.

ENERGY RESERVES IN *PERKINSUS MARINUS* INFECTED AND UNINFECTED OYSTERS. **V. G. Encomio,*** **S. Stickler,** and **F. L. Chu,** Virginia Institute of Marine Science, College of William and Mary, Gloucester Point, VA 23062.

Energy reserves are important for fueling gametogenesis and providing energy during stress. Variation in energy reserves between oyster populations is primarily attributed to environmental differences. Additionally, genetically based intraspecific variation in reproductive timing of oysters has been found in previous studies. By that same notion, intraspecific variation in seasonal patterns of energy storage may also possess some degree of genetic influence. We are examining intraspecific variation in biochemical composition and the effects of parasitism on energy reserves in eastern oysters (*Crassostrea virginica*) and the role of energy reserves in tolerance to Dermo disease. Comparisons will be made between hatchery raised progeny from presumably genetically distinct oyster populations. These oysters represent geographically disparate populations (Gulf of Mexico and Chesapeake Bay) and populations (Gulf—Oyster Bayou, Hackberry Bay, Grande Terre; Chesapeake—Tangier Sound, Choptank River, Lower Rappahannock; Hatchery strain—CrosBred) exhibiting variation in tolerance to the protozoan parasite *Perkinsus marinus*. These oyster stocks have been deployed at two sites within the Chesapeake Bay where Dermo disease, but not MSX, is known to occur. Glycogen, lipid (total lipids, polar and neutral lipids), and protein contents, will be measured in tissues of individual oysters and correlated with changes in shell height, condition index, and *Perkinsus marinus* body burden. Preliminary measurements of recently deployed oyster stocks show similar glycogen contents between various oyster stocks ($p = 0.13$). However, as intensity of *P. marinus* enzootic periods increases, we expect to see both between and within population variation in disease response. Other preliminary measurements of adult oysters showed that total lipid contents in heavily infected oysters decreased compared to non-infected, light and moderately infected oysters.

SPECIES DESIGNATION AMONG SYMPATRIC OYSTERS *CRASSOSTREA ARIAKENSIS*, *C. GIGAS*, AND *C. SIKAMAEA*. **Elizabeth A. Francis,*** **Kimberly S. Reece,** and **Standish K. Allen,** Virginia Institute of Marine Science, College of William and Mary, Gloucester Point, VA, 23062; **Patrick M. Gaffney,** College of Marine Studies, University of Delaware, Lewes, DE 19958.

Little has been published about the distribution or population genetic structure of the Asian oyster *Crassostrea ariakensis*. This species, found sympatrically with *C. gigas* and *C. sikamaea*, is believed to be distributed throughout the warm coastal waters of Pakistan, India, China and Japan. However, morphological plasticity and possible hybridization with congeneric species make identifying *C. ariakensis* difficult. Collection of *C. ariakensis* from its presumed distribution is underway for studies aimed at resolving this confusion. Initial sampling efforts have resulted in specimens collected from Japan and several locations in China. Species identification of these samples using two interspecific typing keys yielded conflicting results. Restriction fragment polymorphism (RFLP) data using nuclear and mitochondrial loci suggested that the putative *C. ariakensis* samples actually contained all three sympatric species. Another species identification key based on RFLP analysis of the 16S locus did not support these results. Many individuals typing as *C. gigas* using the first key were identified as *C. ariakensis* with the second key. To provide greater resolution among relationships, ITS-1 sequence data for individuals from each site were compared to sequences of "known" *C. ariakensis*

and *C. gigas*. Phylogenetic analyses using both parsimony and distance indicate the presence of two clades, one including all the samples collected from China and Japan, and another consisting of the "known" *C. gigas*.

EFFECT OF CLAY SUSPENSIONS ON CLEARANCE RATE IN THREE SPECIES OF BENTHIC INVERTEBRATES. **Dane Frank,[1]* Lisa Ewert,[2] Sandra Shumway,[2]** and **J. Evan Ward,[1]** [1]Department of Marine Sciences, University of Connecticut, Groton, CT 06340; [2]Southampton College, Long Island University, Southampton, NY 11968.

Harmful algal blooms pose a threat to areas where fisheries products are a vital part of the economy. Recent attempts are being made, especially in Asia, to displace harmful algal blooms by spraying fine particulate mineral suspensions (e.g. "china clay") over the surface of affected coastal waters. In practice, the particles adsorb onto the surface of the algal cells, promoting coagulation and displacement to the bottom. Very little is known, however, about the impact of this technique on benthic communities and processes. To examine the effects of differing concentrations of china clay, and kaolin on clearance rate, short-term laboratory experiments were performed on three benthic species: the bay scallop (*Argopecten irradians*), the eastern oyster (*Crassostrea virginica*), and the tunicate (*Molgula manhattensis*). Colleagues in Korea furnished us with the china clay used in their field trials. Depletion rate assays were performed using solutions of 10, 100, 1000, or 10,000 mg/l of either china clay or kaolin suspended with *Rhodomonas lens* (1×10^6 cells/ml) in 0.4 μm filtered seawater. Particle concentrations and size distributions were determined before and after experiments, using a Coulter Multisizer. Results showed a decrease in weight specific clearance rates with increasing concentrations of clay or kaolin in all species. Both the oyster and the scallop showed a slight increase in particle clearance at 100 mg/l, and the tunicates showed a similar increase at 100 mg/l. These results strongly suggest that further studies are required to determine the full effects of this clay-coagulation practice on the benthos.

OPEN OCEAN, SUBMERGED LONGLINE CULTURE OF THE BLUE MUSSEL IN NEW ENGLAND: A FIRST-YEAR PROGRESS REPORT. **Ray Grizzle,** Jackson Estuarine Laboratory, University of New Hampshire, Durham, NH 03824; **Richard Langan,** The Cooperative Institute for Coastal and Estuarine Environmental Technology, University of New Hampshire, Durham, NH 03824.

The blue mussel, *Mytilus edulis*, has supported a substantial aquaculture industry in New England for over 20 years. The predominant method is bottom culture in shallow, nearshore waters. The present project is aimed at assessing the development of suspension culture techniques in open ocean waters of New England, and involves spat/seed collection and growout. Spat collection experiments conducted in 1998 and 1999 showed wide spatial and temporal variability in set densities, but adequate numbers were caught in spring/early summer both years near the mouth of the Piscataqua River, NH. Major problems encountered were overgrowth of the collectors by tubularian hydroids and apparent stunting of the seed at high mussel densities. Sufficient seed to stock >700 m of socking material were obtained in 1998. These mussels were deployed to the submerged longline, which is constructed of 2.8 cm diameter "polysteel" rope, on 2 July 1999. The longline is located 10 km offshore from Portsmouth Harbor, NH in about 60 m of water. Mussel size and environmental conditions at the site have been monitored monthly since July. Overall, the mussels averaged 1.0 mm shell growth/wk from 2 July through 9 November. Based on growth data thus far, a total time for spat set to harvest could be substantially less than 2 yr.

HOW HAVE THE WARNING LABELS AND NEGATIVE PUBLICITY ASSOCIATED WITH *VIBRIO VULNIFICUS* IMPACTED DEMAND FOR GULF OF MEXICO PRODUCED OYSTERS? **Walter R. Keithly Jr.*** and **Hamady Diop,** Center for Coastal, Energy, and Environmental Resources, Louisiana State University, Baton Rouge, LA 70803.

Vibrio vulnificus, a naturally growing bacteria that thrives in the warmer waters of the Gulf of Mexico, has been linked to numerous illnesses and deaths from the consumption of raw shellfish harvested from these waters. While ingestion of this bacteria is harmless to the vast majority of the shellfish consuming public, consequences can be extreme for those individuals with compromised immune systems. California, in response to eight confirmed *vibrio* related cases between 1985 and August 1990, five of them fatal, mandated as of 1 March 1991, that all restaurants and stores selling raw Gulf of Mexico oyster product post warning signs that would advise consumers of the potential adverse health effects associated with consumption of the raw Gulf of Mexico oyster product. This state's action, the first of its kind in the nation, was followed shortly thereafter by other states, including Louisiana and Florida. The purpose of this paper is to examine whether the warning labels and associated negative publicity surrounding the issue impacted the demand for the Gulf of Mexico produced product. To accomplish this objective, the Gulf of Mexico dockside oyster price was estimated using standard econometric techniques for the 1981–97 period based on quarterly data. The results suggest that the dockside price was significantly impacted with the overall extent of the impact depending on season.

DEVELOPMENT OF A DEFINED MEDIUM FOR CELLS OF THE EASTERN OYSTER *CRASSOSTREA VIRGINICA*. **Yanli Li** and **Jerome F. La Peyre,*** Department of Veterinary Science, Louisiana State University, Baton Rouge, LA 70803, USA.

A variety of commercial culture media supplemented with fetal bovine serum have been used to maintain oyster cells in primary cultures. Although certain ingredients found in oyster plasma are often added to these commercial media there have been limited attempts to evaluate the benefits of these ingredients to oyster cells and to optimize their concentrations. Moreover, no culture medium has yet been developed specifically for cells of oysters or other bivalve molluscs. This is surprising since it is well known that formulation of a culture medium based on the biochemical composition of insect plasma in 1956, was a major development that facilitated the establishment of numerous insect cell lines. The objective of this study was therefore to formulate and optimize a defined culture medium for oyster cells.

Using a basal medium optimized in a previous study, the effects of more than 30 ingredients on primary ventricle cell cultures were first evaluated individually over a broad range of concentrations. Evaluation was done by comparing cellular metabolic activity and by observing the morphology and contractility of cultured cells. In a second set of experiments, the combined effects of selected ingredients were determined using a statistical optimization approach based on a Plackett-Burmann statistical design. A defined medium (LA-2) was then formulated by supplementing our basal medium with all beneficial ingredients and the merit of LA-2 was determined by comparing it to commercial L-15 medium (Leibovitz) which has most been used to culture oyster cells. Results indicated that LA-2 was far superior to L-15 for maintaining oyster cells in primary cultures. We are now testing a number of supplements to further improve a medium for culturing oyster cells.

VEINED RAPA WHELKS (*RAPANA VENOSA*) IN THE CHESAPEAKE BAY: CURRENT STATUS AND PRELIMINARY REPORTS ON LARVAL GROWTH AND DEVELOPMENT. **Roger Mann** and **Juliana M. Harding,** Department of Fisheries Science, Virginia Institute of Marine Science, Gloucester Point, VA 23062.

Since the initial discovery of the Veined Rapa Whelk (*Rapana venosa*) in the Lower Chesapeake Bay in June 1999, over 650 adult specimens have been donated to the Virginia Institute of Marine Science (VIMS) Rapa Whelk research program. Continuing efforts to map the whelk's distribution in the Lower Chesapeake Bay indicate no new range extensions and a relatively constant population in the lower James River, Hampton Bar, and Ocean View/Little Creek regions. During the summer of 1999, VIMS' broodstock animals laid over 500 egg masses with egg laying activity beginning in May and continuing through August. Egg masses and the resulting larvae were successfully cultured through metamorphosis. Recently settled young *Rapana* have a wide range of dietary capabilities and will feed on local fauna including barnacles, oyster spat, mussels, and *Macoma*. Growth post settlement can be quite rapid. The oldest juveniles from the 1999 larval cultures reached total shell lengths in excess of 20 mm within 4 months of metamorphosis and settlement.

RELEASE OF MUCOPOLYSACCHARIDES BY BIVALVED MOLLUSCS AND THEIR CONTRIBUTION TO THE PRODUCTION OF TRANSPARENT EXOPOLYMER PARTICLES (TEP) IN NEAR SHORE WATERS. **Michael P. McKee,*** **J. Evan Ward,** and **Lisa M. Milke,** Department of Marine Sciences, University of Connecticut, Groton, CT 06340; **Bruce A. MacDonald,** Department of Biology, University of New Brunswick, Saint John, N.B., Canada, E2L 4L5.

In the marine environment, the presence of large, discrete, transparent exopolymer particles (TEP) has been directly correlated with the flocculation of phytoplankton, detritus, and other particles into aggregates known as marine snow. Phytoplankton and bacteria have both been shown to contribute to the production of TEP, via the exudation of precursor sticky mucopolysaccharides. Little is known, however, about other potential sources of mucins that could lead to TEP formation. The purpose of this research is to identify other potential sources of TEP.

Many benthic suspension feeders utilize mucus-coated structures to capture and transport food particles. Recent endoscopic studies have shown that these suspension feeding processes are accompanied by changes in the cohesive nature of mucins. Hydration of mucins may occur when mucus-coated feeding structures are exposed to ambient waters. The solubilized mucopolysaccharides could then be transported out of the pallial cavity and into open waters. In addition, rejection of pseudofeces, a mucous particle matrix, from the mantle cavity may also serve as a source of additional TEP production. The working hypothesis of our research is that bivalves and other suspension feeders release significant amounts of mucins into the surrounding water, and that this material enhances TEP production through physico-chemical processes, resulting in increased flocculation of particles. Concentrations of TEP in both the laboratory and field setting are determined using an Alcian Blue staining technique and quantified using a spectrophotometer. Preliminary field data from the Long Island Sound and Bermuda suggest that the presence of TEP, above background levels, is correlated with proximity to blue mussel (*Mytilus edulis*) beds and other suspension feeders, respectively. Additional laboratory experiments are being conducted in order to quantify the production of TEP by bivalves under controlled conditions.

IMPROVED PROCEDURE TO COUNT *PERKINSUS MARINUS* IN EASTERN OYSTER HEMOLYMPH. **Amy D. Nickens,*** Aquaculture Research Station, Louisiana State University Agricultural Center, Baton Rouge, LA 70820; **Eric Wagner** and **Jerome F. La Peyre,** Department of Veterinary Science, Louisiana State University, Baton Rouge, LA 70803.

Perkinsus marinus infection intensity in *Crassostrea virginica* can be quantified without sacrificing the oyster by determining the density of the parasite in hemolymph samples. The hemolymph assay involves several steps, (1) the enlargement of the parasites in Ray's fluid thioglycollate medium (RFTM), (2) their subsequent isolation from blood cell debris and (3) their quantification after staining with Lugol's solution. However, each step has yet to be optimized. The objective of this study was to improve the procedure for counting *P. marinus* in oyster hemolymph. We examined changes in the number and size (diameter) of isolated parasites as a result of (1) adding different volumes of RFTM (0.2 ml, 1 ml, 5 ml and 25 ml), (2) adding supplements (lipid and oyster extracts) to RFTM, (3) adding five types of FTM (e.g., with and without agar or beef extract) (4) adding different numbers of hemocytes (10^5, 10^6 and 10^7 cells). We also evaluated the use of sodium hydroxide (NaOH) to digest cellular debris and facilitate cell counting.

Our most significant finding was that incubation in RFTM supplemented with lipid (code liver oil) caused parasites to reach a significantly larger size (26 μm) than did incubation in RFTM supplemented with oyster extract (17 mm) or saline control (11 mm). We also found that the absence of agar from fluid thioglycollate medium greatly simplified sample processing without affecting the number or size of parasites. It was clear from this study that simple modifications of the standard hemolymph procedure could be made to improve counting of *P. marinus* in oyster hemolymph.

TIDAL FLUCTUATION IN PHYTOPIGMENT CONCENTRATIONS AND SEDIMENT LOAD AT A MANILA CLAM, *RUDITAPES PHILIPPINARUM*, FARMING GROUND. **Katsuyuki Numaguchi,*** National Research Institute of Fisheries Science, 6-31 Nagai, Yokosuka, Kanagawa 238-0316, Japan.

Fluctuations of the quantity of fractionated particles of suspended solid and phytopigment (chlorophyll *a* and phaeo-pigments) concentrations in the bottom sea water and sediment with the tidal rhythm was investigated at the Kikuchi River estuary of a Manila clam, *Ruditapes philippinarum,* farming ground. Chlorophyll *a* in the bottom seawater increased at flood and high tide, and decreased at ebb and low tide. Fine particles in the bottom seawater had a high ratio of chlorophyll *a* (10–100 μm; 59%) and phaeo-pigments (1.2–10 μm; 59%). Chlorophyll *a* in the sediment increased from flood to high tide, and decreased from ebb to low tide. Fine particles in the sediment had a high ratio of chlorophyll *a* (1.2–50 μm; 64%) and phaeo-pigments (1.2–50 μm; 66%). Phytopigment contents contained in the digestive diverticula of Manila clams increased from the flood to the high tide, and phytopigments contained in the digestive diverticula of Manila clam were almost all phaeo-pigments (78–98%).

These results suggest that phytoplankton are supplied from offshore to the estuary with the tide during flood to high tide periods. And during these periods, phytopigments in the Manila clam digestive diverticula increased rapidly. These results indicate that most of algal diet to Manila clams may be supplied from offshore to the estuarine Manila clam farming ground. Further, there is a high quantity of fine particles chlorophyll *a* and phaeo-pigment contained in the bottom seawater and sediment at the estuary of the Manila clam farming ground.

1999 OREGON STATE UNIVERSITY STUDIES CONDUCTED ON THE EUROPEAN GREEN CRAB, *CARCINUS MAENAS*, SUPPORTED BY OREGON SEA GRANT. **Kelly Palacios,*** Department of Marine Resource Management; **Sylvia Yamada, Laura Hauck,** and **Alex Kalin,** Zoology Department; **Chris Hunt,*** Environmental Science Department, Oregon State University, Corvallis, OR 97331-2914.

Since the arrival of the European Green crab, *Carcinus maenas,* in San Francisco Bay in 1989, there has been widespread concern for the potential impact on Western Pacific estuarine communities and shellfish industries. With these concerns in mind we set out to determine a few of the limits, impacts, affects and differences in invasion history to this aquatic nuisance specie. With the arrival of this crab predator also came the arrival of a new prey handling technique to the *Littorines* of Oregon, supported by lab studies and field studies using tethered lines. Lab studies were also conducted on preference, handling and consumption on a number of bivalves, including commercially important species. Trapping and lab predation studies suggest that *C. maenas* may be limited by adult native Red Rock crab, *Cancer productus.* Measures of molt rates and frequency suggest that *C. maenas* may reach its terminal molt stage at a younger age in Oregon estuaries than it has throughout it's native range, or in previous invasions. Each of these efforts were supported by Oregon Sea Grant.

THE REPRODUCTIVE CYCLE OF CAPTIVE FEMALE GOLDEN KING CRAB, *LITHODES AEQUISPINUS.* **A. J. Paul*** and **J. M. Paul,** University of Alaska, Institute of Marine Science, Seward Marine Center, Seward, AK 99664.

The predicted increase in carapace length (CL) for adult females (CL 104 to 157 mm) was expressed by the equation; New CL (mm) = Initial CL (0.97) + 10.21; (r^2 = 0.91). Increases in CL averaged 5% (SD = 2%) for egg bearing females. The smallest and largest egg bearing females in our collections were typically 120 mm and 150 mm respectively. Using the CL growth

equation a 120 mm female would require 5 molts to reach 150 mm and produce one clutch with each molt if she survived that long.

The egg clutches hatched asynchronously with some hatching during every month of the year. An average of 31 days passed between the time the first and last larvae in a clutch hatched. After a female's eggs hatched she did not molt until an average of 194 days had passed. Soft shell females extruded eggs about 2 to 3 days later. The incubation period averaged 302 days. There was considerable variability in the duration of each of these growth and reproductive events because they could occur at any time of year with different thermal conditions. Typically females required 530 days, or 1.4 years, to complete a reproductive cycle in the laboratory.

OYSTER RESTORATION IN CHESAPEAKE BAY: EFFECTS OF OYSTER DENSITY ON THE ASSOCIATED BENTHIC COMMUNITY. **K. T. Paynter,** Chesapeake Biological Laboratory and Department of Biology, University of Maryland, College Park, MD 20742 USA.

Restoration of shellfish beds has at least two obvious applications: commercial and ecological. While the commercial value of restored areas to the shellfish industry may be obvious, the ecological value of restored shellfish beds has been difficult to quantify. Oyster bar restoration in Chesapeake Bay has only recently begun in earnest. In 1995 and 1996 10 acres of oyster "bottom" was restored with 1 million hatchery produced oyster spat per acre. In 1997 and 1998, additional areas were restored in the Choptank, Patuxent and Chester Rivers. Underwater videography has been employed to examine the differences in the benthic communities associated with high (2 million/acre) and low (250,000/acre) density oyster plantings. Initial results show that high density planting results in significantly higher numbers of individuals and species associated with the reef. Anemones, barnacles, hooked mussels, gobies and blennies are among the inhabitants of recently restored reefs. In contrast, low density plantings or shells alone harbored relatively few individuals and fewer species as well. Oyster density appears to have a substantial and important effect on the reef community that develops within and around restored reefs.

CARDIOREGULATORY NERVES ARE NOT THE SOURCE OF TEMPERATURE-INDUCED HEART RATE MODULATION IN THE AMERICAN LOBSTER (*HOMARUS AMERICANUS*). **S. M. C. Schreiber*** and **W. H. Watson III,** Dept. of Zoology, University of New Hampshire, Durham, NH 03824.

Lobsters are known to express behavioral responses to temperature, including both temperature avoidance and a preferred temperature range. However, the physiological effects of temperature that may influence these behaviors are not well understood. Previous research on the effects of temperature on the heart of the lobster indicates that some source of temperature-induced heart rate modulation exists in intact animals. The cardioregulatory nerves, which control many of the heart responses, had been assumed to be the source of this modulation. However, as temperature responses are often long-term and neurotransmitters are usually associated with short-term responses, a different source for this modulation was hypothesized. To eliminate the possibility that the cardioregulatory nerves were controlling the heart rate response to temperature, the effects of temperature on the heart rates of lobsters with lesioned cardioregulatory nerves were examined. Lobsters were exposed to a series of 5 °C temperature increases from 5–25 °C. The responses of lobsters with lesioned cardioregulatory nerves were compared to responses of intact lobsters. Over the range of temperatures examined, no significant differences were observed in the responses of lesioned and intact animals. Therefore, it can be concluded that the cardioregulatory nerves are not responsible for the temperature-induced modulation of lobster heart rate.

DERMOWATCH: A NEW TOOL FOR MANAGING *PERKINSUS MARINUS* DISEASE IN EASTERN OYSTERS, *CRASSOSTREA VIRGINICA*. **Thomas M. Soniat,*** Department of Biological Sciences, Nicholls State University, Thibodaux, LA 70310; **Enrique V. Kortright,** Kortright Corporation, 102 Allendale Dr. Thibodaux, LA 70301; **Sammy M. Ray,** Department of Marine Biology, Texas A&M University at Galveston, Galveston, TX 77553.

A website called DermoWatch has been established (*www.blueblee.com/dermo*) to track the progression of *Perkinsus marinus* (= *Dermocystidium marinum*) in Galveston Bay and allow users from other locations to also calculate a time to a critical level of disease (t_{Crit}). Data on water temperature (T) and salinity (S), initial level of disease and oyster length are entered. An embedded model converts measured weighted incidence (WI) values and the critical WI to parasite number (a WI of 1.5 is considered critical), calculates a rate of change (r) of the parasite population using measured values of T and S, and solves for t_{Crit} by simulation. Estimates of t_{Crit} from Galveston Bay and other areas should support decisions concerning transplanting infected oysters to lower salinity areas, harvesting heavily-infected populations early, and diverting freshwater into high-salinity estuaries.

GROWTH, MORTALITY, AND DEFENSE AGAINST *PERKINSUS MARINUS* IN EASTERN OYSTERS, *CRASSOSTREA VIRGINICA*. **S. M. Stickler,* V. G. Encomio, F.-L. Chu,** and **S. K. Allen, Jr.,** Virginia Institute of Marine Science, College of William and Mary, Gloucester Point, VA 23062.

The restoration of Eastern Oyster (*Crassostrea virginica*) populations can be accelerated with the development of strains resistant to Dermo disease, caused by the protozoan parasite, *Perkinsus*

marinus. To date, this has meant the slow, methodical approach of selectively breeding oysters that have survived repeated exposure to the parasite. By identifying effective defense mechanisms in surviving individuals or populations, however, we can develop markers that will expedite the production of resistant strains. This study uses a suite of assays to measure potential cellular and humoral defense mechanisms within and between distinct geographic oyster populations. Wild oysters believed to be resistant to Dermo were harvested from both the Gulf of Mexico (Louisiana) and Chesapeake Bay. Hatchery spawned and raised progeny of these oysters have been placed in floats at two sites in both Chesapeake Bay and the Gulf. We are currently comparing growth and mortality and sampling animals to assess variation in defense capability between stocks. Assays include: hemocyte counts and hemocyte killing of *P. marinus* cells, and protein levels, hemolymph lysosomal enzyme levels, and protease inhibitor levels. All assays are correlated against an optimized body burden fluid thioglycollate media assay for *P. marinus* prevalence and intensity for each individual oyster. We are also examining physiological fitness of animals grown out in Chesapeake Bay to determine the effects of parasitism on energy reserves in oysters and the role of energy reserves in tolerance to Dermo infection.

WESTERN REGIONAL AQUACULTURE INDUSTRY SITUATION AND OUTLOOK REPORT: A SHELLFISH PERSPECTIVE. Derrick R. Toba* and **Kenneth K. Chew,** Western Regional Aquaculture Center, School of Fisheries Box 357980, University of Washington, Seattle, WA 98195-7980.

A survey to estimate the aquaculture production in the twelve western states was conducted for the Western Regional Aquaculture Center (WRAC). The western region includes Alaska, Arizona, California, Colorado, Idaho, Montana, Nevada, New Mexico, Oregon, Utah, Washington and Wyoming. This study was designed to 1) provide production estimates for all species cultured, and identify the states in which they are produced; 2) provide information on the current status of each industry and estimate future (year 2002) production; and 3) determine product forms sold by the producers and provide the average selling price. Information on finfish (salmon, trout, catfish, tilapia and others), shellfish (oysters, mussels and others), aquatic plants and nonfoodfish were collected for the survey.

Total aquaculture production in 1997 for the western region was 140,000 pounds, which was valued at $161,000. The three leading states in 1997 aquaculture production, Washington (58.3 million pounds), Idaho (43.5 million pounds) and California (30.5 million pounds), combined to produce over 94.5% of the total weight. California ($70.6 million), Washington ($46.7 million) and Idaho ($33.7 million) combined to account for 93.7% of the total value of 1997 aquaculture production in the western region.

Of the total 1997 western region aquaculture production, 55.1 million pounds (39.4%) and valued at $38.5 million (23.9%) were shellfish. Five states reported commercial shellfish aquaculture harvests and sales: Alaska, Arizona, California, Oregon, and Washington. In each of the coastal states, oysters comprised the majority of shellfish production. Other species harvested included mussels, clams, scallops, abalone, crawfish, and shrimp. Trends over the past ten years were analyzed for each state and species.

APPLICATION OF UNDERWATER TIME-LAPSED VIDEO TECHNOLOGY TO OBSERVE KING AND TANNER CRAB BEHAVIOR IN AND AROUND COMMERCIAL CRAB POTS. Donn Tracy, Alaska Department of Fish and Game, Commercial Fisheries Division, 211 Mission Rd. Kodiak, AK 99615.

Observations of crab behavior in and around crab pots actively fished for extended soak periods have recently been made possible by the advent of an autonomous underwater video recording system. A built-in microprocessor allows time-lapse video event programming for observations over an unlimited time span. During a pilot study in 1998, red king crabs in Bristol Bay, Alaska were observed entering and egressing five pots over soak periods ranging between twenty-four and seventy-two hours. In the 1999 Bering Sea snow crab fishery observations spanning similar soak periods were made in four commercially fished pots. Future application of this prototype system holds promise for gaining insight into numerous aspects of crab behavior and the performance of commercial pot gear.

THE GENETIC ASSESSMENT OF AN "ENHANCED" BAY SCALLOP POPULATION: DO HATCHERY SCALLOPS PRODUCE SUCCESSFUL RECRUITS? Ami E. Wilbur,* Department of Biological Sciences, University of North Carolina-Wilmington, Wilmington, NC 28403; **William S. Arnold** and **Theresa M. Bert,** Florida Marine Research Institute, 100 8th Ave S.E., St. Petersburg, FL 33701.

Restoration and enhancement of shellfish populations have become increasingly more common as overfishing, habitat degradation and disease decimate wild populations. Numerous techniques have been employed (ie. open seeding of juveniles, spawner sanctuaries, habitat rehabilitation) to mitigate for these losses but the relative success of such techniques is often difficult to evaluate. As part of Florida's ongoing effort to enhance bay scallop populations on the Gulf Coast, we have implemented an extensive genetic monitoring program to assess the impact of the planting of hatchery stocks on local recruitment. We have developed an array of genetic markers (mtDNA, microsatellites and introns) that can function as a "genetic tag" for the enhancement stocks, and allow unambiguous identification of the progeny of the outplanted scallops. Preliminary analysis of post-enhancement recruitment, assessed using spat collectors indicates no substantial numerical im-

provement over pre-enhancement recruitment rates. Genetic evaluation of the post-enhancement population is ongoing. Restriction fragment length polymorphism (RFLP) data based on a 833bp mtDNA fragment reveals a slight increase (3%) in the wild population of a rare haplotype that was abundant in the hatchery stocks, suggesting some contribution of hatchery scallops to the post-enhancement wild population.

TRACKING FECAL SOURCES IN DRAYTON HARBOR. **Rob Zisette,**[1] **Walter T. Trial,**[1] and **Mansour Samadpour,**[2] [1]Herrera Environmental Consultants, 2200 Sixth Avenue, Suite 601, Seattle, WA, USA 98121. [2]Dept. of Environmental Health, University of Washington, PO Box 357234, Seattle, WA, USA 98195.

Drayton Harbor, located at the Washington/British Columbia border, was closed in 1995 to commercial oyster farming and recreational shellfish harvesting due to the presence of high levels of fecal coliform bacteria. Sources of fecal contamination were believed to include discharge of municipal sewage in urban areas, failing septic systems, poor animal management practices in rural areas, and discharges from seafood processors and live-aboards in local marinas. A genetic fingerprinting technique was employed in 1998 to determine which sources were most significant and should be the focus of watershed management efforts. *Escherichia coli* were isolated from oyster tissue samples and from water samples collected on four occasions from two urban streams, one rural stream, and four locations in the harbor.

Using the polymerase chain reaction (PCR) technique, DNA from these *E. coli* were compared to *E. coli* DNA from known fecal sources. Percent matches between known and unknown fecal sources were used to assess the relative contribution of the various fecal sources to streams, marine waters, and oysters in the Drayton Harbor watershed. Human fecal sources were only identified in the rural stream, and livestock were the primary fecal source to this stream. Pets and waterfowl were the only fecal sources identified for the urban streams. Identified sources to marine waters and oysters included waterfowl, livestock, seals, and seafood processing wastewater. Watershed management recommendations included improvement of livestock management practices, correction of septic system failures, treatment of seafood processing wastewater, and public education.

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ABSTRACTS OF TECHNICAL PAPERS

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CONTENTS

HEALTH MANAGEMENT OF THE AMERICAN LOBSTER. **Robert C. Bayer, Deanna L. Prince,** and **Maya A. Crosby,** University of Maine, 22 Coburn Hall, Orono, Maine 04469-0014, USA; **Ben Tall** and **Seynabou Fall,** JIFSAN, USFDA, Washington DC 20204 USA; **Michael B. Loughlin,** Department of Bio-systems Science and Engineering, University of Maine, Orono, Maine 04469, USA.

The American lobster, *Homarus americanus*, is subject to several health problems that appear during post-harvest storage and transport. Major sources of post-harvest losses include gaffkemia ordered tail', ciliated protozoan disease, shell disease, and vibriosis and other types of Gram-negative bacterial infections.

Catastrophic losses of lobsters have been most consistently associated with gaffkemia. Infection results when the bacterium *Aerococcus viridans* breaches the integument through wounds. A fatal sepsis is the ultimate outcome of infection, with the onset of mortality dictated by temperature. Gaffkemia is presently monitored by individuals in the lobster industry, with a simple hemolymph culture technique that uses syringes pre-loaded with a selective medium. Lobsters in storage can be treated for gaffkemia with a feed that contains oxytetracyline. Industry use of this feed has greatly reduced associated mortalities.

Ciliated protozoan disease is also associated with some lobster mortality in storage. The causative organism is *Anophryoides haemophila*, which invades lobster tissues and hemal spaces through perforations of the integument. Acute infections are typically characterized by the presence of large numbers of ciliates freely swimming in the hemolymph, and are readily detected by microscopy. Mortality may be due to tissue destruction and loss of hemocytes produced by invading ciliates, or may be due to secondary invaders.

Shell disease also contributes to some market losses in long-term storage facilities. Erosion and necrosis of the exoskeleton not only make lobsters unattractive to the consumer, but also prone to weakness and mortality. Causative factors of shell disease are not conclusively established, but bacteriological examinations indicate that shell lesions are principally associated with bacteria of the genera *Vibrio, Pseudomonas,* and *Aeromonas*. There is also a strong relationship between shell disease and lobster source, and a possible link to lobster nutrition.

Lobster health problems related to *Vibrio* spp. and other Gram-negative bacterial pathogens are apparently increasing in significance. Recent, high mortalities in some Maine lobster pounds have been associated with a strain of Gram-negative bacteria identified as *Vibrio fluvialis*. A simple hemolymph culture test may be useful in screening for infections of this type. Environmental or other etiological factors may also be important in this type of infection. Lobsters with this syndrome are weak and lethargic. A recent study to characterize biochemically and genetically 19 different isolates obtained from diseased lobsters indicated that the isolates were highly susceptible to a variety of antibiotics tested. However, resistance to erythromycin was observed in 6 of the strains. These organisms have a strict temperature growth requirement and are halophilic. Analysis by pulsed field gel electrophoresis revealed 5 highly related subgroups; one strain could not be typed. Strains were found to possess multiple plasmids suggesting that plasmid carriage is found in these strains. However, the role of plasmids in pathogenesis is unknown. Further analysis of the crude preparation showed that it was not a cell-associated protease; indicating the expression of putative adherence factors. A tissue culture assay showed that polymyxin B lysates obtained from cells could cause elongation of Chinese hamster ovary cells, implying the presence of a putative enterotoxin. Small, irregularly distributed, spike-like, electron-dense deposits were observed on individual cells analyzed with an Alcian blue-lysine electron microscopy staining method. These same cells were observed to form clusters of various sizes, held together by similar spike-like structures, interdigitating between the cells. In contrast, unstained, control cells either were barren of all surface structures or displayed a continuous, fine, lace-like coating of extracellular material. These results suggest expression of either a capsule or an array of surface glycoprotein structures.

DISEASES IN SPINY LOBSTER HOLDING IN NEW ZEALAND. **Benjamin K. Diggles,** National Institute of Water and Atmospheric Research Ltd, PO Box 14-901 Kilbirnie, Wellington, New Zealand.

In recent years a significant amount of research has been conducted in New Zealand investigating methods of culturing two species of spiny lobsters, *Jasus edwardsii* and *Jasus verreauxi*. This research and recent legislative changes have lead to the establishment of a small scale commercial mariculture industry based on grow-out of *J. edwardsii* pueruli collected from the wild. Various disease agents have contributed to morbidity and mortality in each of these species, especially during the early stages of development and refinement of rearing techniques. Mortalities of puerulus and juvenile *J. edwardsii* in experimental holding facilities were due to the invasive fungus *Haliphthoros* cf. *milfordensis* and secondary vibriosis. Fouling of gills of *J. edwardsii* juveniles with a thin, septate fungi, a filamentous *Leucothrix*-like bacteria, free living nematodes and ectocommensal ciliates were probably due to poor water quality and system design in lobster rearing systems utilising recirculated seawater. Chronic, low level mortalities of adult *J. edwardsii* in experimental holding tanks were associated with symptoms of swelling, a condition termed Turgid Lobster Syndrome (TLS). Bacteria isolated from lobsters displaying TLS included *Vibrio harveyi* and *V. splendidus* 1, however the aetiology of TLS remains undetermined at present. A small number of moribund adult *J. edwardsii* in a dietary experiment presented pathological symptoms reminiscent of necrotizing hepatopancreatitis. The crustacean pathogen *Vibrio harveyi* was isolated from moribund phyllosomas of *J. verreauxi* exhibiting luminous vibriosis during an acute mortality event in an experimental culture facility. The gross signs of each disease and some suggestions for their prevention and control are described.

HEALTH ASPECTS IN NORWEGIAN LOBSTER STOCK ENHANCEMENT: PRINCIPLES AND PRACTICE. **Knut E. Jørstad, Øivind Bergh,** and **Kari Andersen,** Institute of Marine Research, Bergen, Norway. C/- CSIRO Marine Laboratory, GPO Box 1538, Hobart, Tasmania 7001.

Unless special precautions are taken, large scale releases into the environment of artificially propagated organisms can represent increased risks of spreading diseases in wild populations. In the government funded research program (PUSH) on sea ranching and stock enhancement conducted in Norway from 1990 to 1998, disease testing of broodstock and juveniles was required before permission for release was given (salmon). In the case of the enhancement project of a local stock of European lobster, *Homarus gammarus*, at the Kvitsøy islands, all information available was associated with the lobster disease Gaffkaemia caused by the pathogen *Aerococcus viridans*. This disease is commonly known in American lobster, *Homarus americanus*, but had spread to Europe possibly through commercial import of live specimens. In Norway it was first reported in imported American lobster in 1976, and extensive investigation in Norwegian wild populations in 1981–1984 concluded that the pathogen was not endemic in Norwegian waters. Several cases of the disease were reported in the 1990s at Kvitsøy, but analyses of the berried animals used as broodstock in the enhancement project revealed no pathogens and the activities were conducted according to schedule. The fish disease problems in the Norwegian aquaculture industry have required a more comprehensive legislation and a new law was established from 1998 and regulated all kinds of diseases in farmed and wild organisms including the marine environment. All new plans for establishing lobster hatcheries for stock enhancement and farming will be carefully evaluated by veterinary authorities.

REVIEW OF CRUSTACEAN IMMUNITY. **Kenneth Söderhäll,** Department of Comparative Physiology, Evolutionary Biology Center, University of Uppsala, Villavagen 6, 752 36 Uppsala, Sweden.

Crustaceans lack immune memory and have therefore to rely on innate immune reactions. One such reaction is the clotting process which is very efficient and rapid and consists of a clotting protein present in plasma and a transglutaminase in the blood cells. The clotting protein has been cloned and belongs to the vitellogenin superfamily of proteins. Clotting proteins have also been purified from shrimp and they are very similar in properties to that of crayfish. Another innate immune defence process is the so called proPO-system which is a non-self recognition system and which upon activation by microbial products generates several factors which will aid in the elimination of foreign particles or parasites. This system has been studied in greatest detail in freshwater crayfish and most of the proPO-components have been purified and cloned. Recently, we have also been able to clone some proPO-components from a shrimp, *Peneaus monodon*.

Cellular immune reactions are important in defence, and two communicating proteins have been isolated and cloned: beta-1,3-glucan and peroxinectin. Both of these proteins are associated with the proPO-system and of great surprise was the finding that peroxinectin had a functional peroxidase domain but peroxidase activity was not involved in the cell adhesion activity of peroxinectin and instead a KGD motif was found to be of importance. Recently antibacterial peptides have been characterised in shrimp and they have been named penaeidins.

CELLULAR RESPONSE TO INJURY IN SPINY LOBSTERS. **Brian Jones,** Fisheries WA, 3 Baron-Hay Court, South Perth, Western Australia, 6151.

This paper presents a review of the cellular defense mechanisms of spiny lobsters. These mechanisms can be divided, for convenience, into three broad groupings: maintenance of exoskeleton integrity; foreign agent recognition, inactivation and elimination from the internal organs; and repair of damage by toxins. Cellular defense mechanisms are dependent on circulating hemocytes and phagocytes, fixed phagocytes and fibrocytes. The process or processes by which these cell types are generated and mature in the animal have not yet been adequately described. In addition, attention has only recently focused on the way in which cellular defence responses are influenced by environmental stress and by the nutritional and moult status of the lobster. These are areas of critical importance to animal husbandry and production in aquaculture. While rapid advances are being made in the understanding of humoral defense mechanisms of crustaceans there are still large gaps in our understanding of the cellular components of the system in spiny lobsters.

TECHNIQUES FOR ENUMERATION AND MORPHOLOGY OF HEMOCYTES IN WESTERN ROCK LOBSTER (*PANULIRUS CYGNUS* GEORGE). **Jeff Jago** and **Bob Dunstan,** School of Biomedical Sciences, Curtin University, GPO Box U1987, Perth, Western Australia 6845; **Japo Jussila,** Institute of Applied Biotechnology, Kuopio University, Kuopio, Suomi-Finland; **Louis H. Evans,** Aquatic Science Research Unit, Muresk Institute of Agriculture, Curtin University, GPO Box U1987, Perth, Western Australia 6845.

A review of anticoagulant strategies for the enumeration and differentiation of total hemocytes counts (THC) in western rock lobster samples collected in various field conditions will be presented. Anticoagulant criteria required preservation of morphological characteristics and arresting of clotting and cell adhesion processes for a minimum period of 24 hours will be described as will the characteristic features of hemocyte types found in *Panulirus cygnus*.

CAN COMPROMISED CONDITION EXPLAIN EARLY MORTALITIES IN SPINY LOBSTER CULTURE? **Andrew G. Jeffs.** National Institute of Water and Atmospheric Research Ltd, 269 Khyber Pass Road, Newmarket, Auckland, New Zealand.

The lifecycle of spiny lobsters involves a long larval period that can last for over two years in some species. Planktonic lobster larvae metamorphose to nektonic pueruli, which make their way into shallow waters to settle and later moult to become benthic juveniles. The distance travelled by the pueruli of many species is estimated to be in the order of tens of kilometres, a journey taking up to several weeks. Interestingly, the puerulus in many species appears to be non-feeding, relying entirely on reserves accumulated during the larval phase. Recent research has indicated that stored lipid is critical for fueling the onshore movement and subsequent moulting of the puerulus. Furthermore, some of this research suggests that a proportion of puerulus may be bereft of energy stores upon settlement. This may greatly affect their subsequent chances of survival by preventing development to the moult or exposing them to increased disease risks as a consequence of a lowered immune response. This possibility was investigated at a commercial aquaculture facility where high mortalities were experienced among pueruli taken from the wild as seed stock. Biochemical techniques previously developed for assessing condition in pueruli were used for samples of live, moribund and dead lobsters sampled from the aquaculture facility. The results of this study confirm the importance of lipids to the post-settlement development of puerulus and suggest that the mortalities experienced at the facility were not related to depleted lipid reserves.

MEASURES OF CONDITION IN DIETARY STUDIES ON WESTERN ROCK LOBSTER POST-PUERULI. **Elena Tsvetnenko, Jeremy Brown,** and **Louis H. Evans,** Aquatic Science Research Unit, Muresk Institute of Agriculture, Curtin University of Technology, GPO Box U1987, Perth, Western Australia 6845.

A nutritional study has been conducted for nine weeks on western rock lobster post-pueruli fed either fresh mussel diet (D1) or one of four artificial diets, two in moist (D2 and D3) and two in dry (D4 and D5) pelleted form. Artificial diets were designed in CSIRO Division of Marine Research, Queensland. The formulation and chemical composition of the diets was proprietary information and not supplied.

Growth rates and condition indices were determined for all treatment groups. Growth rates were expressed as average daily gain and specific growth rate. At the commencement of the experiment, moisture content of digestive gland and tail muscle, and hepatosomatic and muscle-somatic wet and dry indices were determined. At the end of the experiment animals from each treatment were examined for the same parameters. In addition, at the end of the experiment, hemolymph was extracted from animals fed D1 and D2. Total number of hemocytes and percent of granular cells were determined in hemolymph samples.

Lobsters fed the natural mussel diet grew significantly faster than those fed the artificial diets. Changes in digestive gland and muscle indices indicated deterioration of animals' condition during the course of the trial in all treatments. This deterioration was more pronounced in lobsters fed artificial diets compared to lobsters fed mussel diet. While there were no significant differences between growth rates in animals fed artificial diets, significant differences were observed in several of the condition indices. The hemolymph parameters, total hemocyte count and proportion of granular cells were in agreement with tissue indices. The use of condition indices for assessing lobster nutritional and health status will be discussed.

TAIL ROT IN SOUTHERN ROCK LOBSTERS (*JASUS EDWARDSII*). **Ruth E. Reuter,** Veterinary Pathology Services, PO Box 445, Glenside, South Australia 5065; **Michael Geddes,** Department of Environmental Biology, University of Adelaide, Adelaide, South Australia 5005; **Louis H. Evans,** Aquatic Science Research Unit, Muresk Institute of Agriculture, Curtin University of Technology, GPO Box U1987, Perth, Western Australia 6845.

Tail rot has been identified as a continuing problem in captive lobsters kept in holding facilities to maximise return on investment. Chitin-destroying bacteria have been identified overseas in lobsters held in groups over winter. In South Australia the condition has been seen during the summer period from December to April. As part of another study on nutrition, samples were collected in March 1999 from five groups of lobsters being fed different diets and held in varying locations on the South coast. Lesions on the tail and/or claw were identified in 11 animals. Histopathology done on 7 samples showed inflammation often associated with cracks and fissures in the overlying chitin. Of four samples cultured, *Vibrio alginolyticus* was cultured from all samples, while *Plesiomonas shigelloides* also obtained from one of the samples. *V. alginolyticus* and *Aeromonas hydrophila* were cultured from similar lesions in lobsters in holding cages in March 1998. These organisms are commonly present in marine and estuarine environments. However they have been associated with skin damage, ulcers, anemia, and tail and fin rot in finfish, and mortality after handling in eels. In the situation described, handling and holding of the lobsters, in association with elevated water temperatures, could be predisposing to invasion of damaged tissue by organisms such as *V. alginolyticus*.

A STUDY OF DISEASES IN CULTURED PHYLLOSOMA LARVAE AND JUVENILES OF SOUTHERN ROCK LOBSTER (*JASUS EDWARDSII*). **Judith Handlinger, Jeremy Carson, Arthur Ritar,** and **Bradley Crear,** Tasmanian Aquaculture and Fisheries Institute, University of Tasmania, Hobart, Tasmania.

Cultured phyllosoma larvae and juveniles of southern rock lobster (*Jasus edwardsii*) were monitored for disease for almost two years. No major disease outbreaks and no specific pathogens have been seen in either age group, though relatively few were examined. A variety of external fouling organisms and occasional deeper invasions were seen, which have contributed to either sporadic losses or to background low level mortality. Fouling consistently involved adhered *Leucothrix*-like bacteria that provided a habitat for a complex microbiota consisting of clumps of smaller bacteria, stalked peritrich ciliates, *Chilodonella*-like flagellates, amoebae, and occasional fungi. Bacteria isolated from animals held in culture, and possibly associated with focal degeneration and adhesion of the exoskeleton, particularly in appendages, included *Flavobacteium* species and mixed *Vibrio* species including *V. anguillarum, V. alginolyticus* and *V. tubiashii*, all recognised pathogens of several aquatic animals. Histological examination suggested these bacteria were largely in small granulomas in appendages (gills in juveniles), or in the hepatopancreas tubules. *V. harveyi* was once isolated from newly collected small juveniles with digestive tubule degeneration and occasional granulomas with visible bacteria. Isolation of bacteria from hemolymph of juveniles was rare, and there was no histological evidence of bacteremia in either age group. Heavy fouling and gill tip necrosis reflected water quality (high ammonia, low dissolved oxygen) and was largely controlled by manipulation of environmental conditions.

INDICATORS OF STRESS IN THE HEMOLYMPH OF THE WESTERN ROCK LOBSTER (*PANULIRUS CYGNUS* GEORGE). **Glen W. Davidson** and **Patrick T. Spanoghe,** Centre for Food Technology, Queensland Department of Primary Industries, C/- School of Biomedical Sciences, Curtin University of Technology, GPO Box U1987, Perth, Western Australia 6845; **Brian D. Paterson,** Centre for Food Technology, Queensland Department of Primary Industries, Queensland ,19 Hercules street, Hamilton, Queensland 4007.

The western rock lobster *Panulirus cygnus* supports a large, sustainable commercial fishery in Western Australia. This species is wholly sub-tidal, but experiences repeated episodes of exposure to air during post-harvest. The effect of air exposure on the lobsters is confounded by handling/disturbance and temperature fluctuation. The cumulative effect of these stressors is observed as a reduction in the muscle tone, vigour and responsiveness of the lobsters. However, depending on the severity and duration of the stressful encounter, these symptoms may reflect anything from temporary exhaustion to permanent damage resulting in imminent death. Traditional methods of grading lobsters for various product forms depend on visual assessments of animals and, for the above reasons, can be misleading. The purpose of the present work was to identify physiological indicators of stress that are associated with future morbidity and mortality during post-harvest handling. After implementing practices which maximise survivorship, useful physiological indicators can be used to assess the sublethal effects of alternative handling practices. Focusing on hemolymph constituents, a range of metabolites, electrolytes, enzyme activities, proteins, and hemolymph blood gas and acid-base parameters were measured in lobsters undergoing actual or simulated post-harvest handling treatments. These factory-based experiments were supported by closely controlled laboratory experiments. Baseline levels of parameters of interest were determined in free-ranging lobsters in the field by divers equipped with SCUBA, and also in acclimation studies in the laboratory. These studies also provide information regarding how the parameters vary with moulting activity. In this paper we discuss normal ranges for potential stress indicators in *P. cygnus*. The response dynamics of hemolymph variables to specific stressors are also presented along with some interpretation of their physiological significance.

PHYSIOLOGICAL PROFILES AND VIGOUR INDEX OF LOBSTERS (*PANULIRUS CYGNUS*) DELIVERED TO PROCESSING FACILITIES. **Patrick T. Spanoghe,** Centre for Food Technology, Queensland Department of Primary Industries, C/- School of Biomedical Sciences, Curtin University of Technology, GPO Box U1987, Perth, Western Australia 6845; **Philip K. Bourne,** School of Biomedical Sciences, Curtin University of Technology, GPO Box U1987, Perth, Western Australia 6845.

In Western Australia, lobsters delivered to processing premises are routinely subjected to a grading procedure, aiming at selecting from the catch the animals best suited for live export marketing. This assessment is essentially based on a range of criteria referring to the general morphological appearance of the animals such as the size, the colour of the shell, the number of missing appendages but also to the behavioural appearance of the animals, that is essentially the intensity of their somatic responses to physical stimulation. This paper presents and discusses the results of a study comparing the physiological profiles of a sample of lobsters assessed and graded for vigour, with reference to a selection of behavioural observations. It presents the results of an analysis aimed at identifying the physiological variables best correlated to a vigour index.

THE USE OF HEMOLYMPH CHEMISTRY IN CONDITION ASSESSMENT OF THE SOUTHERN ROCK LOBSTER (*JASUS EDWARDSII*). **Richard J. B. Musgrove,** SARDI Aquatic Sciences, 2 Hamra Ave, West Beach , South Australia 5024.

A study was carried out to develop a condition index for the characterisation of temporal and spatial changes in condition and growth for the southern rock lobster, *Jasus edwardsii*.

Serum protein concentration has been accepted as a coarse indicator of condition, because of its correlation with muscle mass, particularly percent muscle mass, which has been shown to decline on starvation and to change with moult stage. The problem has been in the integration of the moult cycle-dependent body composition with the measurement of condition. The moult cycle and condition are inextricably linked. Simply measuring tissue or hemolymph composition is of doubtful utility if the moult stage is not accurately known. This is made especially difficult if intermoult (C_4) is long, as is the case with many lobsters. There is no way of distinguishing between those at the beginning and those at the end of this stage. This is particularly important as muscle and storage tissue accumulation continue from ecdysis until late premoult.

In this paper I present and discuss a method for condition assessment of commercial-sized lobsters based on the serum protein: tissue relationship and blood pigmentation. The latter may be broken down into eight stages, four of which occur during intermoult. It is shown that both percent dry tissue and total dry tissue may be predicted using a combination of the two measurements. The use of blood lipid in prediction of moult increment is also discussed.

POST-HARVEST HANDLING STRESS IN WESTERN ROCK LOBSTER: HEMOCYTES' POINT OF VIEW. **Japo Jussila,** Institute of Applied Biotechnology, Kuopio University, Kuopio, Suomi-Finland; **Elena Tsvetnenko** and **Louis H. Evans,** Aquatic Science Research Unit, Muresk Institute of Agriculture, Curtin University, GPO Box U1987, Perth, Western Australia 6845; **Jeff Jago,** School of Biomedical Sciences, Curtin University, GPO Box U1987, Perth, Western Australia 6845.

The hemocyte response in western rock lobster (*Panulirus cygnus*) to conditions of post-harvest handling stress was investigated in a series of studies carried out either under practical conditions or in a research laboratory. Total hemocyte counts (THCs) and differential hemocyte counts (DHCs) were studied along with other indicators of stress. Lobsters were sampled in different stages of post-harvest handling or as part of specially planned experiments, with the hemocyte sample (200 μL) collected from either ventral or pericardial sinus into precooled Na-Cacodylate anticoagulant and later analysed for THCs and DHCs. The minimum sample number required to give significant differences between treatment groups was observed to be 10, while statistical methods suggested a sample size of 18. The results suggested that THCs and DHCs could be used as stress or condition indicators under various post-harvest handling conditions. THCs were indicative of physical disturbance, loss of condition and starvation, while air exposure as sole stressors seemed to cause less changes in the numbers of circulating hemocytes. The changes in DHCs were more prone to indicate changes in the lobsters' physical condition, and the decreasing hyalinocytes proportion seemed to correlate with bacteremia. It was concluded that between 4 and 8 × 10^6 cells/mL of THCs could be considered to be an undisturbed background level, while THCs lower than that could indicate worsening condition and point of mortality and levels higher than that could indicate different levels of stress. The indications of stress or change in the lobsters' physical condition could be seen both in THCs and DHCs. These could be used as tools in measuring the effects of post-harvest handling on western rock lobsters, especially in conjunction with other stress parameters.

COLDWATER LOBSTER HEALTH: A NORTH AMERICAN PERSPECTIVE. **Richard J. Cawthorn,** Lobster Health Research Centre, Atlantic Veterinary College, University of Prince Edwards Island, Charlottetown, Prince Edwards Island, Canada C1A 4P3.

In North America, one of the largest traditional surviving fisheries involves the coldwater clawed Amercian lobster *Homarus americanus*. However, post-harvest losses are conservatively estimated at 10–15%, representing an economic impact of $50–75 million annually. The mandate of the Lobster Health Research Centre is to apply the principles of veterinary medicine to the post-harvest sector of crustacean fisheries and to crustacean aquaculture. The primary task is to define what constitutes a healthy lobster, and subsequently to maintain or enhance the health status of lobsters. Important infectious diseases in confinement situations include "bumber car" disease caused by the ciliate *Anophryoides haemophila*, gaffkemia caused by the bacterium *Aerococcus viridans*, and shell disease associated with bacterial species of *Aeromonas, Pseudomonas* and *Vibrio*. Additional factors reducing lobster health are improper handling, exposure to adverse weather, inappropriate bait, inadequate nutrition and environmental stressors. Lobster health surveillance requires knowledge of ecosystem health, development of lobster databanks, and interaction at all levels of the fishery to enhance lobster health management.

IMMUNOLOGICAL MEASURES OF STRESS IN SPINY LOBSTERS (*PANULIRUS CYGNUS* GEORGE). Louis H. Evans, Seema Fotedar, Japo Jussila, Shannon McBride, and **Elena Tsvetnenko,** Aquatic Science Research Unit, Muresk Institute of Agriculture, Curtin University of Technology, GPO Box U1987, Perth, Western Australia 6845.

Methodology for six different assays of immunological stress parameters, total hemocyte counts (THC), differential hemocyte counts or % granular cells (%gran), antibacterial factor (ABF), phagocytic capacity (phag), clotting time (clot) and hemolymph bacterial colony counts (CFU/ml; bact) were developed and applied in studies of stress responses in the spiny lobster *Panulirus cygnus*. An investigation of the influence of handling procedures and other minor disturbances on the levels of immune parameters in lobsters held in the laboratory showed that THC increased & phag decreased within minutes of exposure to a handling stressor (placement in a foam box and shaken for 1 min and every subsequent 30 min for 2 h), clot decreased two hr after stressor exposure while ABF and %gran were unaffected by minor stressors. Bacterial levels in hemolymph in the test lobsters were high at the beginning of the experiment and showed no significant variation following stressor exposure.

In two simulated transport and live shipment trials (November 1998 and March 1999) in which lobsters were held either in water (submerged), in air (humid air) or in a spray system (spray) at ambient temperature (November 22 °C; March 26 °C) for 6 h in five enclosed compartments fitted with either flowthrough (flow) or recirculating (recirc) water systems (flow submerged, recirc submerged, humid air, flow spray and recirc spray) all parameters in hemolymph taken at the completion of the 6 h exposure period studied showed a consistent pattern of variation between treatment groups with the exception of THC. Mean values of ABF, bact and clot in lobsters held in humid air or in spray systems were higher and mean %gran was lower than values observed in the submerged lobsters. The patterns of variation in ABF, bact, clot and %gran in the five different treatments mirrored the pattern of survival of lobsters following tank storage and simulated shipment with surviving lobsters showing lower ABF, bact and clot and higher %gran than lobsters which died or were weak at packout or following simulated live transport. Hemolymph did not clot in a small proportion (3.9–10.2%) of lobsters from all five treatments in the first trial and in the flow submerged, recirc submerged and humid air treatments in the second trial. The percentage of lobsters with hemolymph which didn't clot was significantly higher in the flow spray and recirc spray treatments in the March trial (25.5% and 20.8% respectively). Autopsies performed on weak lobsters removed from factory tanks within one to several days after the simulated transport showed that the mortalities were likely to have been caused by bacterial infections in the bladder and the antennal glands. The results suggest that THC and phagocytic capacity show rapid responses to postharvest handling stressors, clotting time initially decreases and then increases while bacteremia and the levels of ABF increase following stressor exposure. The application of these variations in immune parameters to the determination of the stress status or health status of postharvest lobsters will be discussed.

A PRELIMINARY EVALUATION OF THREE HEMOLYMPH TESTS TO ASSESS HEALTH STATUS IN TROPICAL ROCK LOBSTERS (*PANULIRUS ORNATUS*). John H. Norton, Naomi Levy* and **Kelly Field,** Oonoonba Veterinary Laboratories, Queensland Department of Primary Industries, PO Box 1085, Townsville, Queensland 4810.

Three hemolymph tests were evaluated on small numbers of tropical rock lobsters *Panulirus ornatus* as possible indicators of health status. These included a phenoloxidase test, a red blood cell (RBC) agglutination test and an antibacterial test. They were conducted on both clinically normal and sick adult lobsters. Highly significant differences ($P < 0.01$) were obtained for the phenoloxidase and RBC agglutination tests. Although no significant difference was obtained for the antibacterial test, further work with larger numbers of lobsters may prove otherwise. These pilot experiments strongly suggest that further experimentation with these three tests on both clinically normal and sick adult rock lobster hemolymph would be productive.

RESPONSES OF SPINY LOBSTER (*JASUS EDWARDSII*) FED L-CARNITINE AS A DIETARY SUPPLEMENT UNDER TEMPERATURE AND STARVATION STRESS. Manel Dias-Wanigasekera, Jean Pierre Dufour, and **Philip V. Mladenov,** Department of Marine Science, University of Otago, PO Box 56, Dunedin, New Zealand.

The effects of l-carnitine supplemented in a squid based diet formulated for juvenile *Jasus edwardsii* were investigated in three feeding experiments. Growth and survival responses were collectively expressed as the normalized biomass index (NBI). L-carnitine was included at a level of 300 mg/kg. The NBI was 6.7 for lobsters fed a diet supplemented with l-carnitine and fatty acids, in comparison with lobsters fed an unsupplemented diet (NBI = 4.07) or mussel (6.06) as food. Survival was positively correlated to the NBI ($r2 = 0.64$). After starvation for two weeks, 98% of the lobster juveniles fed l-carnitine survived, whereas a survival of 70% was obtained for lobsters fed other diets. In a subsequent experiment, exposure to high temperature stress (32 °C/3h), caused an immediate mortality of 17% in l-carnitine fed animals and 48% in juvenile lobsters fed an unsupplemented diet. Exposure to low temperature stress (0 °C/6h) produced an immediate mortality of 45% in juvenile lobsters fed mussel and 12% to 18% in animals fed various unsupplemented feeds. All l-carnitine fed animals survived. Independent of the diet, all animals showed growth retardation following temperature shock. Growth promoting effects of l-carnitine were seen immediately

after administration via feed, whereby the animals fed this compound displayed an acceleration of the moulting process. Supplementation of diets with regulated amounts of l-carnitine over short periods improves growth on a short-term basis, and this improvement could be used to advantage in starter diets. L-carnitine also improves physiological resistance of lobsters to stressful conditions often met with during long-term holding, aquaculture and live transport. The biological activity of l-carnitine in rock lobsters seems to be between that of a growth promoter and a vitamin compound. However, care needs to be exerted so that the period of feeding and level of supplementation are carefully monitored.

POST-HARVEST HANDLING IN THE WESTERN ROCK LOBSTER FISHERY. Stephen Hood, MG Kailis Group of Companies, 50 Mews Road, Fremantle WA 6160.

The western rock lobster, *Panulirus cygnus*, forms the basis for one of the world's largest lobster fisheries with a catch value in excess of A$250 million annually. The fishery supports over 600 catcher vessels along almost 1000 km of coastline and offshore to a depth of 250 m. This large geographical fishing area and the highly seasonal nature of the industry creates unique post-harvest handling considerations for both the wild capture and processing sectors of the industry. Currently, only some 30% of the total catch is exported as live product, largely as a consequence of the seasonal and geographical distribution of the catch. This presentation deals with post-harvest handling considerations from capture through to final export as a live product, as well as addressing some of the other processing techniques. The presentation also deals with potential and actual problem areas in the post-harvest sector, applied research that has been undertaken to overcome these problems, and the potential for future research to further enhance the value of the catch.

IDENTIFYING INDICATORS OF STRESS DURING POST-HARVEST HANDLING OF WESTERN ROCK LOBSTERS (*PANULIRUS CYGNUS*). Brian D. Paterson, Centre for Food Technology, Queensland Department of Primary Industries, Queensland, 19 Hercules street, Hamilton, Queensland 4007; **Patrick T. Spanoghe** and **Glen W. Davidson,** Centre for Food Technology, Queensland Department of Primary Industries, C/- School of Biomedical Sciences, Curtin University of Technology, GPO Box U1987, Perth, Western Australia 6845.

Lobsters are stressed when a factor, sometimes called a stressor, causes their internal physiology to deviate from normal. Currently western rock lobsters, *Panulirus cygnus*, are graded a number of times during post-harvest handling to remove injured or damaged lobsters as well as removing individuals that are considered to be 'weak' on the basis of their posture or responsiveness. These stressed lobsters are singled out because experience shows they are unlikely to survive for long in the factory. Simply knowing that these lobsters are stressed may not be of much practical use. We want to know what it is about particular lobsters that mean that they survive a handling treatment but die subsequently. To find these indicators we subjected large numbers of lobsters to a controlled period of stress, then sampled their blood immediately to measure a number of physiological parameters. The lobsters were tagged and stored in a factory and their fate recorded. The group of lobsters that survived differed statistically from the group that didn't survive in a number of parameters. But examining the frequency distributions of selected parameters highlights how difficult it is to apply one parameter meaningfully to individual lobsters. The distributions of the survivor and non-survivor samples can overlap considerably. However, discriminant analysis, a multivariate technique that uses combinations of parameters to describe the differences between groups can be used to identify the parameters contributing to these discriminant functions and thus provide the stress indicator we seek. Dead lobsters are easy to count, but knowing the key physiological characteristics of lobsters that have been stressed too much and 'exhausted' by a handling treatment provides important feedback on how to change that treatment to minimise losses.

THE EFFECTS OF TEMPERATURE ON THE RESPIRATORY FUNCTION OF THE NEW ZEALAND LOBSTER (*JASUS EDWARDSII*) IN AIR AND WATER. Michelle M. Pritchard and **H. Harry Taylor,** Department of Zoology, University of Canterbury, 58 Brodie Street, Upper Riccarton, Christchurch, New Zealand.

The magnitude and rate of change of oxygen consumption in water following short and long-term temperature changes were quantified using closed box respirometry. Aerial oxygen consumption was measured at different temperatures using a flow-through system and Ametek Oxygen Analyser. On emersion, aerial oxygen consumption decreased at first but progressively recovered during extended emersion. We hypothesized that this increase in oxygen consumption might be due to either: a) drying of the gills in air, leading to improved diffusive conductance of the gills; or b) an increase in the oxygen affinity of the hemocyanin after prolonged emersion. Blood gas analysis (pre- and post-branchial Po_2 and oxygen content) and acid-base analysis (pH and l-lactate concentration of the hemolymph) was carried out at 5 °C, 12 °C and 18 °C in water and in air after various emersion times. Oxygen equilibrium curves were generated from hemolymph taken from lobsters emersed at 12 °C for 4 or 24 h and from aquatic controls. These data do not support an increase in gill conductance but suggest that there was an increase in hemocyanin oxygen affinity following 24 h emersion, compared with the values measured at 4 h emersion and the control values. L-lactate concentration increased during emersion and may be partially responsible for the increase in oxygen affinity following 24 h emersion.

DETERMINATION OF TOTAL PROTEIN IN HEMOLYMPH OF THE WESTERN ROCK LOBSTER (*PANULIRUS CYGNUS* GEORGE) BY REFRACTOMETRY.

Brian D. Paterson, Centre for Food Technology, Queensland Department of Primary Industries, Queensland ,19 Hercules street, Hamilton, Queensland 4007; **Glen W. Davidson** and **Patrick T. Spanoghe,** Centre for Food Technology, Queensland Department of Primary Industries, C/- School of Biomedical Sciences, Curtin University of Technology, GPO Box U1987, Perth, Western Australia 6845.

Research on western rock lobsters (*Panulirus cygnus*) has shown that refractometry is a simple non-destructive field technique for assessing the hemolymph protein concentration and hence the tissue mass or "condition" of rock lobsters (Dall, W. 1975. *J. Exp. Mar. Biol. Ecol.* 18:1–18). However, a conversion from refractive index (RI) to protein concentration has not been published for this species. Hemolymph samples were obtained from the pericardial sinus of lobsters via the arthrodial membrane between the posterior margin of the carapace and the abdomen using disposable hypodermic syringes. In order to convert RI values to protein concentrations, the refractive index of a number of hemolymph samples were measured at ambient temperature using a Shibuya S-1 salinometer calibrated at ambient temperature with distilled water. Total protein concentrations were determined colourimetrically by the biuret method. Comparing the data gave the following regression equation:

Total protein (mg/mL) = (5402.398 × RI) - 7214.877, r^2 = 0.947, n = 28

This analytical method represents a simple, and useful way to obtain important information about the condition of rock lobsters entering commercial handling and transport. The measurement involves a straightforward physical phenomenon and it is probably not surprising that the conversion equation obtained in this study is similar to that derived from a study of the American lobster (Leavitt, D.F. & Bayer, R.C. 1977. *Aquaculture* 12:169–171). These conversions may be a satisfactory method of estimating blood protein concentration in other large marine crustaceans. If large numbers of measurements are to be made for other species using this method, it is relatively easy to establish a calibration to ensure the accuracy of the technique.

INVESTIGATIONS OF BACTEREMIA IN SPINY ROCK LOBSTERS.

Seema Fotedar, Anne Barnes, and **Louis Evans,** Aquatic Science Research Unit, Curtin University of Technology, GPO Box U1987, Perth, Western Australia 6845; **Mike Geddes,** Department of Environmental Biology, University of Adelaide, Adelaide, South Australia 5005; **Ruth Reuter,** Veterinary Pathology Services, PO Box 445, Glenside, South Australia 5065.

Bacteremia was studied as part of various laboratory based and factory based stress trials in the spiny lobster, *Panulirus cygnus* and in a dietary trial in southern rock lobster, *Jasus edwardsii*. Bacteremia was assessed in three different ways: 1) based on percent prevalence; 2) based on mean colony rank (ranging from 0 (0 CFU/ml) to 13 (>8.45 × 10^3 CFU/ml)); and 3) based on mean colony count (CFU/ml). Under unstressed conditions very low bacteremia levels were observed (rank 0–1). Minor stressors such as handling disturbance and repetitive sampling led to increased prevalence of bacteremia in *Panulirus cygnus*. In two simulated transport and shipment trials significant levels of bacteremia were observed. The frequency distribution of colony ranks showed larger numbers within ranks 0–5 and rank 13 and few in between.

The dietary trial on *Jasus edwardsii* involved on-site sampling following collection from cages and repetition of sampling after air freight (approx 1 h) and 16–24 h storage in a cold room at 4 °C. Bacteremia results were consistent with those of *Panulirus cygnus*, with increased prevalence of bacteremia in the 24 h samples. The dietary treatment had no significant effect on the level of bacteremia.

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ABSTRACTS OF TECHNICAL PAPERS

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CONTENTS

PROGRESS IMPLEMENTING A PLAN TO MONITOR AND CONTROL POPULATIONS OF THE EUROPEAN GREEN CRAB (*CARCINUS MAENAS*) IN WASHINGTON COASTAL ESTUARIES. **Elizabeth M. Carr** and **Brett R. Dumbauld**, Washington State Department of Fish and Wildlife, P.O. Box 190, Ocean Park, WA 98640.

The European green crab (*Carcinus maenas*) was first noted in Washington State waters during the summer of 1998, having steadily progressed northward from California where its presence in San Francisco Bay was first recorded in 1989. The two distinct year classes present appear to have resulted from strong larval recruitment events and not from new introductions. The response to this bio-invasion was rapid and resulted in a plan to monitor and control green crab populations in Willapa Bay and Grays Harbor, Washington which was approved and implemented in 1999. Monitoring results suggest the same two year classes are still present. Although mating couples and females with viable eggs have been found, no new recruitment has been noted to date. Catch per unit effort has declined as the population has apparently spread out in both estuaries making control efforts potentially more difficult. Trapping methods continue to be refined as the primary control technique.

DISTRIBUTION OF *VIBRIO PARAHAEMOLYTICUS* IN PUGET SOUND OYSTERS, WATER, AND SEDIMENTS—PRELIMINARY RESULTS USING A MOLECULAR METHOD. **Russell P. Herwig** and **Robyn M. Estes**, School of Fisheries, University of Washington, 3707 Brooklyn Ave NE, Seattle, Washington 98105; **Cindy L. Messey** and **Daniel P. Cheney**, Pacific Shellfish Institute, 120 State Ave NE, #142, Olympia, Washington 98501.

Vibrio parahaemolyticus (*Vp*) is a bacterium that is naturally found in estuarine and marine waters around the world. Selected strains of *Vp* may cause food borne illness. This organism can accumulate in filter-feeding organisms, such as oysters. *Vp* may be a problem with oysters that are harvested in the warm summer months. During the summers of 1997 and 1998, several outbreaks of *Vp* gastroenteritis were caused by the consumption of raw oysters harvested from the Pacific Northwest. Federal and state regulatory agencies have established a concentration of 10,000 cells per gram of oyster meat as the level of concern.

During the summer of 1999, oyster, sediment, and water samples were collected from four sites in Puget Sound, Washington. Levels of *Vp* in the various samples were determined using a newly-developed molecular Most Probable Number (MPN) method. Samples were blended, diluted, and inoculated into a series of tubes containing Alkaline Peptone Water (APW). This medium was incubated overnight at 37 °C. Small aliquots of liquid were removed from each incubated APW tube and used in a polymerase chain reaction (PCR) procedure. This molecular method was used to amplify a specific fragment of DNA that is found only in *Vp*. The enumeration of *Vp* in the molecular MPN method was performed within 2 days, a much shorter time compared to the FDA-approved method.

Our results indicated that levels of *Vp* in Puget Sound oysters, sediment, and water were not detectable or very low in June 1999, increased at selected sites in July, and remained elevated in August. Concentrations of *Vp* were much higher in sediment compared to water samples. Although the different Puget Sound sites had comparable water temperatures and salinities during the summer, a site on Hood Canal had much higher concentrations of the potential human pathogen in samples collected during July and August. Interestingly, the site with the highest *Vp* levels had sediments that were very coarse compared to the other sites. The reasons for the elevated levels of *Vp* at particular locations in Puget Sound is not understood.

AN UPDATE ON THE ONGOING OYSTER SUMMER MORTALITY STUDY: MORTALITY OF THE PACIFIC OYSTER, *CRASSOSTREA GIGAS*: HEALTH SCREENING, ENVIRONMENTAL LINKS AND MANAGEMENT OPTIONS. **Daniel P. Cheney, Ralph A. Elston,** and **Brian F. MacDonald**, Pacific Shellfish Institute, 120 State Avenue N.E. #142, Olympia, WA 98501; **Gary N. Cherr, Amro M. Hamdoun,** and **Jacqueline L. Jacobsen**, Bodega Marine Laboratory, Bodega Bay, CA 94923.

This project tests hypotheses relating to mortalities of Pacific oysters on the West Coast. Four sites in Puget Sound experienced limited mortality at all stations through the end of June 1999. By the end of August increasing mortalities of up to 15% between sample dates were seen in several treatment groups. These mortalities paralleled a similar pattern of very high seed oyster mortalities in Tomales Bay. Puget Sound ambient air and water temperatures through late spring 1999 were similar to 1998; however after June, peak air temperatures were below 1998 levels. Dissolved oxygen, pH and salinity levels at all stations remained nominal throughout most of this period. There was a correlation between densities of *Gymnodinium sanguineum*, *Ceratium* spp., *Pseudo-nitzschia* spp. and other dominant taxa with the onset of summer mortality. In addition, moribund oysters had lesions consistent with an acute toxic effect. A biotoxin, most likely of algal origin, was considered as a probable cause. No infectious diseases considered certifiable or reportable by regulatory agencies were observed in these oysters. Investigations into the physiological responses of oysters to stress are now underway to provide further insights into the survival process. We are currently field testing outplants to see if a correlation exists between the biochemical response and the organismal response.

This research is supported by a grant from the National Sea Grant College Oyster Disease Research Program and matching contributions from West Coast shellfish growers.

POPULATION GENETICS OF *PROTOTHACA STAMINEA* AND *MACOMA BALTHICA* IN PUGET SOUND, WA. **Micaela Schnitzler Parker,** School of Oceanography, University of Washington, Campus Box 357940, Seattle, Washington 98195.

Individuals from three populations of *Protothaca staminea* and *Macoma balthica* were examined electrophoretically and scored at 5 allozyme loci. Each of the three populations is located in a different hydrologically-defined basin of Puget Sound, WA. Highly significant differences between the three *Protothaca staminea* populations were found at all 5 loci. However, only at one locus could the *Macoma balthica* populations be differentiated. Genetic distances between the three *Protothaca staminea* populations were determined using both Cavalli-Sforza and Edwards (1967) chord distance and Nei's (1972) genetic distance measures. A genetic similarity dendrogram is presented and discussed in the context of Puget Sound hydrology.

Between two and four of the allozyme loci demonstrated heterozygote deficiencies in *Protothaca staminea*, depending on population. Only one locus was heterozygote deficient in each of the three *Macoma balthica* populations. Implications of these deficiencies, as well as the differences in population differentiation between these two taxa, are presented.

ISOLATION OF NOVEL MICROSATELLITES IN GEODUCK CLAMS (*PANOPEA ABRUPTA*) BY MAGNETIC BEAD HYBRIDIZATION SELECTION. **Brent A. Vadopalas, Patrick T. O'Reilly,** and **Paul Bentzen,** Marine Molecular Biotechnology Laboratory, University of Washington, Seattle, Washington 98105.

Information regarding the genetic population structure of Puget Sound geoduck clams should guide culture and fishery management of this economically valuable species. If population substructure exists, both the avoidance of genetic intermixing caused by aquaculture practices and the re-designation of wild tracts available to fishers may be prudent for genetic conservation. Because of their high variability and their ability to be assayed from extremely small samples, microsatellite DNA markers are generally considered the best tools available for the detection of genetic variation.

Novel di- and tetranucleotide microsatellite loci were developed in geoduck clams by magnetic bead hybridization selection as markers for population genetic analyses. Polymerase chain reaction (PCR) screening of a 155 clone library yielded 82 positive clones. DNA sequencing revealed that 62 of the positive clones contained microsatellite arrays, primarily $(GATA)_n$, some in combination with other tetranucleotide array motifs. Three of the clones contained short $(AC)_n$ arrays. PCR primer sets were designed for 16 candidate loci. Mono- or di-allelic PCR profiles were observed at 10 loci and all were polymorphic. These markers will be used to examine population structure and variance in reproductive success.

Preliminary screening revealed a deficiency of heterozygotes at many loci. If these heterozygote deficiencies are not due to laboratory artifacts, larvae of type parents may be screened in an attempt to ascertain the geoduck clam life history stage(s) associated with these heterozygote deficient loci.

CURRENT STATUS OF THE EUROPEAN GREEN CRAB, *CARCINUS MAENUS*, AND NATIVE CRAB SPECIES IN YAQUINA AND COOS BAYS. **Laura L. Hauck,** Student, Department of Biology, Oregon State University, Corvallis, Oregon 97331; **Sylvia Behrens Yamada,** Department of Zoology, Oregon State University, Corvallis, Oregon 97331.

Four different sampling methods were employed at four sites in Yaquina Bay and five sites in Coos Bay to monitor the progress of the invasive *Carcinus maenas* (CM) population. We also recorded the status of native crab species: *Cancer magister, Cancer productus, Hemigraphus oregonensis, Hemigrapsus nudus,* and *Pachygrapsus crassipes,* in the intertidal zone at the current invasion level.

The first method used at each site was a tethered snail predation line. This method gives the best natural estimation of predation rate at a given location, and allows us to compare predation rates from site to site. Crab species hitting the line are identified by their shell cracking technique when the lines are checked. Trapping followed the first method to verify the identity and presence of crab species. Rock turning was the third method used which yielded information on shore crab species. The last method used was to search for molts of a new year class at the high tide water line.

No recruitment evidence of a CM new year class has yet been found in either bay by an sampling method. Data recorded in 1997 on all crab species was compared to data collected this year.

THE DISTRIBUTION OF THE EUROPEAN GREEN CRAB, *CARCINUS MAENAS*, IN YAQUINA BAY, OREGON. **Chris Hunt,** Environmental Science Department, Oregon State University, Corvallis, Oregon 97331-2914.

Carcinus maenas, first discovered in Coos Bay, Oregon in 1997, was found in at least eight Oregon estuaries in 1998. With the absence of a successful 1999 year class to date, their present population exists of what is believed to be two and three year old crabs. These older, and much larger, crabs are present lower in the estuary than last year, creating the potential for more interactions with the larger native Cancrids. An intensive trapping effort during the summer of 1999 documented the distribution of this older year class of *C. maenas* and other crab species in Yaquina Bay. It appears that *C. maenas* coexists in areas with the native Dungeness crab, *Cancer magister*, but appears to be absent, or limited, in areas suitable for another native crab, the Red Rock crab, *Cancer productus*. Although *C. maenas* appears to be abundant in areas

with fewer of these larger crabs, it appears dense populations of adult *C. productus* may be a primary limiting factor in the lower estuary for this new invasive species.

DEVELOPING A PEST MONITORING PLAN FOR BURROWING SHRIMP: A STEP TOWARDS INTEGRATED PEST MANAGEMENT. **Brett R. Dumbauld,** Washington State Department of Fish and Wildlife, P.O. Box 190, Ocean Park, WA 98640; **Daniel P. Cheney,** and **Brian F. MacDonald,** Pacific Shellfish Institute, 120 State Avenue N.E. #142, Olympia, WA 98501.

Several attempts have been made to develop an integrated pest management (IPM) plan for burrowing shrimp in Washington state coastal estuaries. Burrowing shrimp are an important pest to the oyster aquaculture industry and also cause habitat changes for other benthic organisms in coastal estuaries across the Pacific Northwest. Oyster farmers in Washington State have applied the pesticide carbaryl to control these shrimp on privately owned estuarine tidelands since the early 1960's. A recent IPM feasibility study identified several critical issues that needed to be addressed before IPM could be successfully implemented.

A project to monitor shrimp populations in Grays Harbor and Willapa Bay, Washington was initiated in July 1999 to address some of these issues. Objectives are to: (1) examine and monitor the overall efficacy of burrowing shrimp control measures; (2) follow patterns and rates of shrimp recruitment and survival and compare these with oyster survival and production through typical grow-out cycles on treated and un-treated oyster beds; and (3) use the data collected to develop a long term monitoring plan for burrowing shrimp. Preliminary information on inherent variability in burrow count data and efficacy of the 1999 treatment are presented.

REESTABLISHMENT OF A NATIVE OYSTER: IMPLICATIONS FOR POPULATION DISTRIBUTION AND STRUCTURE. **Patrick Baker,** Department of Fisheries and Aquatic Sciences, University of Florida, Gainesville, FL 32653; **Nora B. Terwilliger,** Oregon Institute of Marine Biology, University of Oregon, Charleston, OR 97420.

The reappearance of the Olympia oyster, *Ostrea conchaphila* (= *lurida*) in Coos Bay, Oregon, raises questions about its population structure and dispersal. On the outer coast from Washington to central California, the Olympia oyster occurs only in certain estuaries. This species went extinct in Coos Bay prior to European settlement, and both deliberate and accidental inoculations prior to 1988 failed to reestablish it. Since 1988, the Olympia oyster has become abundant in Coos Bay, with heavy and regular recruitment. Why did the Olympia oyster become reestablished in 1988, but not previously? From where did it reinvade Coos Bay, and how?

Coos Bay's geographic isolation may prevent reestablishment of Olympia oyster from other populations by larval dispersal, but does not explain the failure of human introductions. Our research suggests, however, that the Olympia oyster has returned partly because of human modifications to the estuary. Incremental deepening of the bay for navigation has "restored" a prehistoric salinity regime, making it similar to modern Olympia oyster habitat elsewhere. Olympia oysters currently occur in the most modified portion of Coos Bay, in which modal winter salinities have increased strongly since at least 1950. If this scenario is correct, habitat limitation, not dispersal, is probably the primary limiting factor for the Olympia oyster in this region. Molecular genetic analysis is underway to determine the source population of the Coos Bay population, and the degree of isolation between Olympia oyster populations. Knowing the source population will also allow us to develop hypotheses for the vector of reestablishment.

GROWTH OF 1997/1998 YEAR CLASS OF THE GREEN SHORE CRAB, *CARCINUS MAENAS*, IN OREGON. **Alex Kalin** and **Sylvia Behrens Yamada,** Department of Zoology, Oregon State University, Corvallis, Oregon 97331.

During the spring of 1997, a strong new year class of *Carcinus maenas* appeared in seven Oregon estuaries. The carapace width of these crabs averaged 14 mm June, 27 mm in July and 45 mm in September 1998. By the summer of 1999, the crabs had reached between 44 and 80 mm in carapace width. The growth of tagged crabs and a molt increment study of captive crabs support these rapid size increases.

Carapace width data collected from Oregon bays suggests that *C. maenas* is growing faster in Oregon than in the North Sea or Maine. Our data supports the theory that *C. maenas* reaches sexual maturity within one year in Oregon, while in the North Sea and Maine sexual maturation may take two to three years. Molt increment data, however, suggests that growth per molt is constant in *C. maenas* populations regardless of geographic location. Regression equations of molt increment data of Oregon specimens showed no statistically significant difference from similar regression equations describing North Sea and Maine populations. The molt increment statistical analyses lead to the inference that *C. maenas* molts more frequently in Oregon than in the North Sea or Maine.

PROGRESS IMPLEMENTING A PLAN TO MONITOR FOR PRESENCE OF THE EUROPEAN GREEN CRAB (*CARCINUS MAENAS*) IN PUGET SOUND, WASHINGTON. **Anita E. Cook** and **Sandra Hanson,** Washington State Department of Fish and Wildlife (WDFW), Point Whitney Shellfish Lab, Brinnon, WA 98320.

A significant population of the European green crab, whose first persistent presence on the U.S. west coast was recorded in

1989 in San Francisco, was first noted in Washington State in 1998 in coastal Willapa Bay and Grays Harbor. The green crab likely arrived in Washington via larval drift on ocean currents. To date no European green crab have been confirmed in Puget Sound.

A large-scale Puget Sound green crab monitoring program was established in 1999, with WDFW as the coordinating agency. The primary aim of this initial phase was thorough geographical sampling coverage of Puget Sound (including the Strait of Juan de Fuca and the San Juan Islands) to maximize the potential of detecting any green crab that might have spread to Puget Sound by larval transport or other means. This was accomplished by enlisting and training various volunteers to set crayfish traps at monitoring sites spread throughout the Puget Sound. Over 15 groups sampled more than 50 monitoring stations in 1999. Participants included non-profit volunteer organizations, shellfish growers, tribes, marine science centers, government agencies, schools, and the general public. In addition to providing information about the potential presence of green crab in Puget Sound, the trapping supplied some general baseline data about populations of small native crab in the sampling areas. In the year 2000 WDFW will focus on increasing the number of sample sites (for higher potential of discovering green crab presence), identifying sites with the highest likelihood for introductions, and examining other green crab detection techniques.

DEVELOPMENT OF A SPECIFIC-PATHOGEN-FREE (SPF) HATCHERY AND NURSERY FOR PRODUCTION OF PACIFIC OYSTER SEED. Chris J. Langdon, Dave P. Jacobson, Ford Evans, Ebru Önal, and **Sean E. Matson,** Hatfield Marine Science Center, Oregon State University, Newport, Oregon 97365.

The Molluscan Broodstock Program (MBP) is sited on the Yaquina Bay, Oregon, and produces Pacific oyster seed for testing at commercial grow-out sites on the West Coast, U.S. in order to identify families with superior yields. In 1998, an adult Pacific oyster from Yaquina Bay was found to be infected with a haplosporidian—later shown to positively react with a DNA probe for Seaside Organism (SSO). In order to prevent exposure of MBP seed to potential haplosporidian infectious agents in Yaquina Bay, seawater supplied to culture systems was filtered to 1 μm micron and subsequently either filtered to 0.22 μm for larval culture or UV-treated (>90,000 μW sec/cm^2) for spat culture.

Growth of larvae and spat in treated seawater was significantly poorer than in non-treated water. Experiments indicated that addition of antibiotics (2 mg/L chloramphenicol alternated with 2 mg/L erythromycin) improved larval growth in cultures supplied with 0.2 μm-filtered water, while addition of 2 mg/L calcium bentonite improved growth of both larvae and spat in treated water. No haplosporidian infections were found in a sample of 168 spat reared in MBP facilities, indicating that the adopted SPF procedures were effective.

INTERIM PERFORMANCE OF PROGENY OF SELECTED BROODSTOCK FROM THE MOLLUSCAN BROODSTOCK PROGRAM. Chris J. Langdon, Dave P. Jacobson, and **Ford Evans,** Hatfield Marine Science Center, Oregon State University, Newport, Oregon 97365; **Mike S. Blouin,** Zoology Department, Oregon State University, Corvallis, Oregon 97331.

The Molluscan Broodstock Program (MBP) was established to improve yields of Pacific oysters on the West Coast, U.S., by genetic selection. Fifty full-sib families were planted in Tomales Bay, California, in October 1996 and the top nine families with the highest yields (meat weight per bag) were identified in July 1997. In 1998, 45 fill-sib families were produced by crossing the top nine families among and within themselves to produce out-crossed and inbred families, respectively. The progeny were planted at Totten Inlet, Puget Sound, Washington, in August 1998. In addition, progeny of non-selected "wild" oysters together with samples of industry-produced seed were planted for comparison. An interim weighing of oysters (before they reached market size) was carried out in June 1999.

Results indicate that the mean yield (live weight per bag) of progeny of selected MBP families was significantly greater (Fisher's PLSD test: $P < 0.05$) than those of both non-selected and inbred families but not significantly different from that of industry seed. Heritability for yield was estimated to be 0.54, indicating that family yields should improve through genetic selection. The poor performance of inbred families underscores the importance of avoiding inbreeding in commercial oyster production.

SHELLFISH HIGH HEALTH PROGRAM. Ralph Elston, Pacific Shellfish Institute, PO Box 687, Carlsborg, WA 98324, **Dan Cheney,** Pacific Shellfish Institute, Olympia, WA.

An oyster high health program was designed and implementation begun. The purpose is to provide a health database for Pacific oysters and to establish high health guidelines for live shellfish producers that would facilitate entry into markets with regulatory requirements for shellfish disease free status. Voluntary implementation of a shellfish high health program by seed producers or other exporters of live shellfish destined for receiving waters should expand markets and facilitate the process of obtaining import permits.

Oyster brood stocks from California, Oregon, and Washington sites were examined to provide 95% confidence of detecting conditions occurring at a 2% prevalence or greater. Nocardiosis was found in 3.2% of oysters from one site while *Mytilicola* infestations were found in several samples at prevalences less than 2%. Ruptured reproductive follicles were found in up to 27% of oysters. Various other non-infectious conditions were found at prevalences of less than 5%. No examples of infectious diseases currently considered certifiable, including haplosporidia, *Mikrocytos mackini*, *Perkinsus* spp., *Bonamia* spp., *Marteilia* spp., unidenti-

fied protistan parasites or histological evidence of viral infections were found.

Components of the proposed voluntary program include a system of health certifications, records and documentation, maintenance of brood stock integrity, hatchery and nursery operations protocols as needed to maintain infectious disease exclusion and a response plan for infectious disease outbreaks.

FECUNDITY STUDY AND PRELIMINARY FIELD TRIAL RESULTS OF THE BUTTER CLAM (*SAXIDOMUS GIGANTEUS*). Amilee Caffey and **Brady Blake,** Point Whitney Lab., Washington Department of Fish and Wildlife, Brinnon, WA 98320.

In 1996, the Point Whitney Shellfish Hatchery began a series of spawning trials on the butter clam, *Saxidomus giganteus*. The main goal of the study was to determine values for a population management model used for clam resources. The first value obtained through this experiment was the smallest clam observed spawning: 29.21 mm male shell length/29.95 female shell length. The second value included overall mean egg production at 2.07 million eggs per female.

This study is a continuation of that work for 1997–1999. Spawning trials included not only the smallest clam observed spawning and overall egg production, but also looked at values such as brood stock holding temperature, algal concentrations during spawning trials, and potential egg production of repeat spawners in one season. Overall mean production for the combined years dropped to 1.07 million eggs per female with the smallest female clam observed spawning remaining at 29.95 mm and the smallest male clam observed spawning at 29.21 mm shell length. Results show that butter clams are repeat spawners with an overall egg production of 1.08 million and were observed spawning at this level up to three times in one month. The highest egg production occurred when the brood stock was conditioned at 10.5 °C. During the spawning trial, the highest egg production also occurred at algal concentrations of 950,000 cells/ml during spawning trials.

Seed from the study was then planted at the Point Whitney beach and sampled survival and growth rates after a two year period. Mean shell length of butter clams at time of plant was 19.62 mm and mean shell length at recovery after two years was 42.34 mm. The average growth of the butter clam was 22.72 mm over two years. Survival rate was 7.10%.

BURROWING RESPONSE OF JUVENILE GEODUCKS (*PANOPEA ABRUPTA*) TO CHANGES IN TEMPERATURE AND SALINITY. Jonathan P. Davis, Clea Barenburg, and **David Pederson,** Taylor Resources Bivalve Hatchery, 701 Broad Spit Road, Quilcene, WA 98376.

Geoduck clams, *Panopea abrupta*, are a newly cultured species and the development of geoduck culture techniques, coupled with out planting methods have not been perfected. Environmental parameters likely have a significant effect on the burrowing behavior of clams which in turn may greatly influence the level of survivorship of newly planted seed.

The burrowing behavior of three size classes of juvenile geoduck clams was measured in response to exposure to a suite of temperature and salinity conditions. Seed were exposed to all combinations of six temperature (8, 11, 14, 17, 20, & 23 °C) and six salinity (20, 22, 24, 26, 28, & 30 ppt) treatments. Three different seed classes were tested; small (4.6 mm mean shell length), medium (7.2 mm) and large (9.5 mm) geoducks. All clams were maintained under common conditions prior to testing burrowing response.

Results indicate that all seed size classes showed maximal burrowing response at median temperatures (11, 14, and 17 °C) and higher salinities (26, 28, and 30 ppt). The response for all size classes indicated a proportionate increase in burrowing rate as conditions neared ambient salinity (30–32 ppt). Size was also a significant factor as large and medium seed demonstrated high burrowing response only between 11 and 14 °C and at higher salinities, and reduced burrowing response at low (8 and 11 °C) and high (23 °C) treatment temperatures. The burrowing response of small seed in all treatments was uniformly higher compared to medium and large seed across all temperature and salinity treatments; however as also seen for large and medium sized cohorts, burrowing behavior at salinities less than 26 ppt. was greatly reduced. Avoiding extremes in temperature and in particular salinities less than 26 ppt. even for short periods of time, may significantly increase overall planting success for culture operations.

POST SETTLEMENT GEODUCK CLAM (*PANOPEA ABRUPTA*) GROWTH AND SURVIVORSHIP IN SAND AND SCREEN-BASED NURSERY SYSTEMS. Jonathan P. Davis and **Clea Barenburg,** Taylor Resources Bivalve Hatchery, 701 Broad Spit Road, Quilcene, WA 98376.

Post settlement growth and survivorship in sand and screen based down-welling silos was examined for geoduck clams in order to assess the viability of different nursery systems during this critical life history stage. Geoduck pediveligers were introduced to three sand-based substrates including fine (500–600 micron) and course (700–800 micron) sands and screened (500–600 micron) dolomite particles. Three screen-based treatments were assessed using density as a dependent variable. Three initial stocking densities tested were: 10, 40, and 80 geoduck plantigrades per cm^2, respectively. All sand-based treatments were set at an initial stocking density of 10 clams per cm^2. All setting systems were rinsed with filtered sea water once weekly over the 25 day experiment. Sea water used for the downwelling setting system was 5 micron filtered and UV treated and set on a recirculation mode with approximately 1.5 L per minute replacement. A variety of cultured algae was fed to the clams on a continuous basis for up to 18 h per day.

Results indicated that all sand-based systems had significantly higher post-set survivorship than any of the screen-based treatments. The following was observed in order of survivorship after 25 days: small-grained sands (6.07 clams per cm^2) < large-grained sands (4.44) < dolomite sands (3.36) < screen-based at 80 clams per cm^2 (0.60) < 10 clams per cm^2 (0.40) < 40 clams per cm^2 (0.33). Growth rate of surviving clams in all of the treatments was similar, although the onset of mortality in sand and screen-based systems showed a tendency for clams to survive to a larger size in the sand-based systems.

A TAG METHOD FOR ESTIMATING THE NATURAL MORTALITY RATE OF GEODUCKS (*PANOPEA ABRUPTA*). **Alex Bradbury, Don P. Rothaus, Robert Sizemore,** and **Michael Ulrich,** Washington Department of Fish and Wildlife. Point Whitney Shellfish Laboratory, Brinnon, Washington 98320.

We tested a tagging method for estimating the instantaneous rate of natural mortality (M) of subtidal geoduck clams (*Panopea abrupta*) at a previously unfished site in Hood Canal, Washington. Divers "tagged" 1128 adult geoducks (>3–4 yrs) in May 1998 by placing thin plastic stakes next to geoduck siphons at a distance of 7.6 cm. Geoducks were tagged within 1 m of three lines running offshore and anchored in depths of −6 m to −23 m MLLW. One year later, divers found 875 of the original 1128 tags remaining in the substrate. Over a 6-day period, siphons were visible next to 856 of the tags. Divers used a venturi dredge to excavate the 19 tags with no visible siphons; 4 of these geoducks were alive, 14 were dead, and one tag had no sign of a living or dead geoduck. The annual survival rate (S) was estimated as $N_1/N_0 = 861/875 = 0.984\ yr^{-1}$ and the corresponding estimate of M was $0.016\ yr^{-1}$ (95% CI = 0.025 – 0.007). This point estimate is lower than the value currently used in Washington's equilibrium yield model ($M = 0.0226$), but is not significantly different. The direct estimate of M makes fewer assumptions than catch curve estimates, and is less expensive.

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COVER PHOTO: Eight day-old larvae of the Pacific oyster (*Crassostrea gigas*) viewed under epifluorescent light (excitation 355–425 nm, emission 525 nm) at ×400 magnification. Larvae were fed on riboflavin-containing lipid spray beads (50 beads/μl) for one hour, followed by a two hour period of feeding on *Isochrysis galbana* (T-ISO) alone. Free riboflavin is evident as a diffuse greenish fluorescence in the guts of larvae while riboflavin crystals present in intact or partially digested SB are evident as bright yellow points. The digestive systems of some larvae also fluoresce red due to the presence of chlorophyll from ingested algae. Average larval shell length = 122 μm. (C. Langdon)

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CONTENTS CONTINUED

JOURNAL OF SHELLFISH RESEARCH

Vol. 19, No. 1 June 2000

CONTENTS

CONTENTS CONTINUED ON INSIDE BACK COVER

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PREVALENCE AND PROGRESSION OF SHELL DISEASE IN AMERICAN LOBSTER, *HOMARUS AMERICANUS*, FROM RHODE ISLAND WATERS AND THE OFFSHORE CANYONS

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ABSTRACT From 1995 to 1999, shell disease in lobsters, *Homarus americanus*, was monitored in research trawl and trap surveys conducted in Rhode Island waters including Narragansett Bay, Rhode Island and Block Island Sound, and the offshore areas of Block and Hudson Canyons. In the inshore population a significant increase in frequency and severity of the disease was documented beginning in 1996, reaching 20% infected by 1999 with over 50% of ovigerous females affected. Shell disease was noted in the offshore lobster population beginning in 1998. A tag-recapture study conducted in Narragansett Bay between 1997 and 1999 followed the change in severity over a 3-year period in the field for 86 individuals. Observed disease pathways point to potential affects for reproductive behavior and mortality. The proportion infected with disease appeared to diminish over the molting period, but subsequently increased in infection rate and shell coverage during the months of September and October.

KEY WORDS: Lobster, shell disease, field observations, tag-recapture

INTRODUCTION

Parasites and disease can be significant factors in population dynamics. There is evidence that disease presents a major force that shapes populations that is as profound as the forces of predation or resource utilization (Anderson and May 1979, Hart 1990). Disease outbreaks have been known to produce significant losses in all life history stages of cultured and wild American lobsters (Bayer et al. 1993).

Shell disease has been studied extensively in relation to high winter mortality rates in commercial lobster pounds where animals are confined in high densities for up to 6 mo in duration. Shell disease is caused by an external infection that occurs when chitinoclastic organisms, including several bacteria and fungal species, attack the exoskeleton. *Vibrio* spp are most commonly found and several other gram-negative bacteria are usually present in necrotic pits. Researchers believe that it is the combined action of these chitinoclastic organisms that interact to cause the general appearance of shell disease. Very little information is available about shell disease in wild lobsters. In the natural environment chitinoclastic bacteria are believed to cause little harm, living in the substrate and on the animal itself (Johnson 1983).

Shell disease lesions typically begin as small dark brown or black pits in the exoskeleton, indicating that the calcified layers have been eroded (Getchell 1989). The blackening is due to the melanization of the necrotic area. In minor cases the non-calcified layers remain intact; however, in more severe cases, inner tissues become damaged. Damage to the exoskeleton is not a prerequisite to shell damage, although it can be a precursor (Stewart 1984). Malloy (1978) was able to experimentally transmit the disease in *H. americanus* after abrasion, however, this has only been shown to occur under degraded or crowded conditions (Getchell 1989).

Bullis et al. (1988) described two general forms of shell disease in red crabs, one associated with injuries that appeared as random localized areas and one that was manifested as bilateral lesions in areas of microscopic sensory organelles on the carapace. Later stages of this latter type were characterized by extensive areas of darkened melanization leading to loss of large areas of the exoskeleton. Sindermann (1991) hypothesized that shell disease was an external indication of some metabolic disturbance that results in the failure of the animal to keep up with chiton deposition. Metabolism can be affected by a number of environmental stressors such as pollutants, anoxia, or physiological changes such as poor nutrition.

Estrella (1991) found that shell disease prevalence in Massachusetts in 1989 was significantly higher in larger lobsters, suggesting an inverse relationship with molting frequency in hard shelled lobsters. Ovigerous females displayed a significantly higher percentage of disease and mature non-ovigerous females displayed higher percentages than males. If the appearance and worsening of shell disease are related to the frequency of molting, then we would expect smaller, immature animals to be less infected than larger, mature animals. We would also expect mature females to be more infected than males or immature females since they carry eggs for up to 9 to 11 mo and may not molt for two years (Waddy et al. 1995). However, if shell disease is a consequence of internal metabolic disturbance, then the new shell may only provide a short respite before the disease reappears.

The recent increase in extensive shell damage in lobsters in Massachusetts, Rhode Island, and Long Island Sound waters has not been previously recorded for wild stocks. The appearance of the disease also coincides with the discovery of a "limp lobster syndrome" in the Gulf of Maine that is causing weakness and mortality of lobsters in pounds (Bayer pers. commun., Maine Lobster Institute) and a large mortality in Western Long Island possibly caused by a paramoeba infection. The contribution of shell disease to natural mortality of lobsters may be significant (Taylor 1948), as are the effects on other life history characteristics such as growth or reproduction. This study describes the

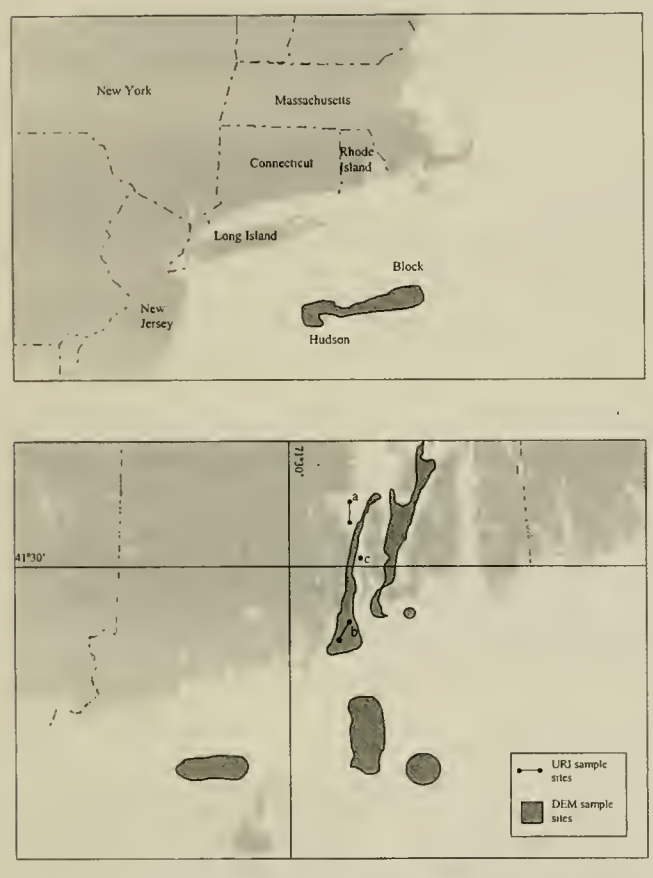

Figure 1. Rhode Island offshore sea sampling areas (above). Location of URI trawl survey sites (a). Wickford (41°34.45'N/71°24.34'W to 41°33.45'N/71°24.34' W) (b). Whale Rock (41°26.55'N/71°25.14W to 41°25.10W/71°25.54'W), and (c) Trap survey Dutch Harbor. Rhode Island Inshore Sea sampling sites (below).

prevalence and progression of shell disease in the lobster population in Rhode Island waters and offshore canyons from 1995 to 1999.

MATERIALS AND METHODS

Shell condition of the American lobster, *Homarus americanus* has been routinely monitored during several state and research surveys in Rhode Island and offshore waters (Fig. 1). The University of Rhode Island (URI) conducted a weekly research trawl survey at two sites (Wickford and Whale Rock) in Narragansett Bay between 1995 and 1999. Sea sampling surveys were conducted monthly from 1996 to 1999 by Rhode Island Department of Environmental Management (DEM) biologists aboard fishing vessels throughout Narragansett Bay, Rhode Island and Block Island Sound, and offshore canyons. Twenty-four inshore trips per year and four offshore trips per year were sampled. During spring, summer, and fall of 1997 to 1999, a weekly trap survey for tag-recapture studies was conducted at three sites (associated with an artificial reef project) in Dutch Harbor in the West Passage of Narragansett Bay, using six commercial traps covered with small mesh at each site.

Lobsters from all surveys were sexed, measured (for carapace length [CL]), examined for molt stage, egg-bearing status, cull status, and shell disease, and those from the URI studies were tagged with a numbered t-bar anchor tag (Floy tag) before being returned to the water. Bottom water temperature was recorded on the URI trawl survey using a Nanson bottle and a thermometer.

TABLE 1.

Rating system used to score shell disease index (SDI) score. Three areas of the lobster were visually examined and assigned a score 0 to 100 corresponding to the percentage of each body section affected. These were added up and assigned a rating from 0 to 4.

SDI	Percentage of coverage of abdomen, claws, and carapace
0	None
1	1–100
2	101–200
3	201–250
4	251–300

The occurrence of shell disease in the URI surveys was noted by drawing a symbol indicating the location and size of the occasional lesion. However, this system was later modified into a shell disease index (SDI) which describes the percentage of covered area in three body areas: carapace, abdomen, and claws. Each body section has a maximum of 100 points for a maximum score of 300 (Table 1). The DEM survey recorded presence and absence of shell disease only.

Each survey was evaluated as proportion infected by sex category and weighted by number captured to obtain proportion infected by year. Differences between years were statistically analyzed using an approximate z test based on normal approximations to the binomial (Ostle and Malone 1988). The URI trawl and trap data were examined for independence of variables using the Spearman Rank Correlation procedure followed by partial correlation analysis (SPSS, Base 10). The Mann-Whitney two-sample test or the chi-square test of independence was applied to examine the effects of variables on disease incidence (Sokal and Rohlf 1981, SPSS, Base 10). A regression analysis was used to test relationships between infection rate and both temperature and proportion soft. Differences in size frequency distributions were evaluated using large sample Kolmogorov-Smirnov tests and a student's

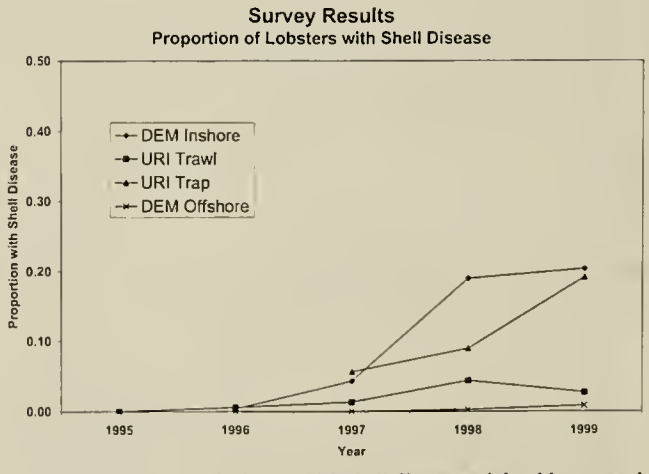

Figure 2. Proportion of lobsters with shell disease weighted by sex and number for each survey (RI DEM inshore sea sampling survey, RI DEM offshore sea sampling survey, URI trawl survey, and Dutch Harbor trap survey).

TABLE 2.

Proportion of lobsters with shell disease and total number sampled (in parentheses) from inshore and offshore areas (RI DEM sea sampling trap data). Significant values are indicated with an asterisk.

Lobster type	Males	Non-ovigerous female	Ovigerous females	Weighted means (z scores)
Inshore				
1996	0.0043 (8,754)	0.00063 (14,163)	0.0142 (3,022)	0.00345
1997	0.0304 (6,216)	0.0125 (10,343)	0.16663 (3,151)	0.0427 (26.43*)
1998	0.0956 (5,325)	0.104 (10,182)	0.4984 (4,438)	0.1895 (46.94*)
1999	0.1023 (6,236)	0.092 (9,806)	0.53 (5,235)	0.203 (3.39*)
Offshore				
1996	0 (12,087)	0 (16,704)	0 (1,019)	0
1997	0 (11,864)	.00005 (18,881)	0 (1,182)	2.96E-05 (0.99)
1998	0.0032 (10,160)	.0031 (14,437)	0.0074 (1,631)	.002415 (8.09*)
1999	0.0056 (11,176)	.0066 (13,622)	0.0522 (1,053)	.00803 (8.93)*

t test was used for evaluating mean sizes. All differences were tested for significance at $\alpha = 0.05$.

For the tag/recapture study, lobsters received a shell disease difference score for each recapture event. Shell disease difference (SDD) was obtained by subtracting the recapture SDI from the previous capture SDI. Results were statistically analyzed using a chi-square test of independence (SPSS, Base 10) using Pearson's chi-square statistic at $\alpha = 0.05$ for effect of size, sex, and season on shell disease outcome score (Sokal and Rohlf 1981).

RESULTS

The presentation of shell disease typically began with small lesions occurring in the epicuticle of the carapace and abdomen that were characterized by erosion and melanization. Many of these lesions affected deeper layers of the cuticle. The site of deepest penetration was an area directly behind the rostrum. In later stages the entire shell became flexible. The last area affected was the claws. Many newly molted lobsters had areas of melanization already present in the new epicuticle.

The DEM inshore survey examined a total of 86,871 lobsters. Total numbers of lobsters from all combined sites showed a significant increase for every year in the proportion of shell diseased lobsters in the inshore areas beginning in 1996, with the highest infection rate of 20% in 1999 (Fig. 2; Table 2). The highest infection rates were for ovigerous females, with over 50% displaying signs of shell disease in 1999. A total of 87,865 lobsters were examined in the offshore canyon survey. This area is beginning to show low infection rates (Fig. 2; Table 2). In 1999 there was an infection rate of 0.8%, with highest rates in ovigerous females (5.22%).

The URI trawl survey examined a total of 18,841 lobsters. Shell disease was first noted in the Wickford upper bay site in October 1995. The first occurrence of shell diseased lobsters in Whale Rock occurred in May 1996. Both URI sites showed a significant increase from 1995, with the highest infection rate in ovigerous females in 1998 (Fig. 2; Table 3). Overall, proportions infected from the trawl survey were much lower than the DEM survey, only reaching 4.35% in 1998. However, infection rates for ovigerous females were high: 52% in Whale Rock and 33% in Wickford.

Shell disease prevalence from the URI trawl survey was positively correlated with site, sex, size, and molt and was negatively correlated with temperature. No correlation was found with cull status (Table 4). Third order partial correlation controlling for temperature, site, and molt status resulted in significant positive correlations between shell disease and sex ($P < 0.001$) and size ($P = 0.028$). There was a very weak relationship between proportion infected and bottom temperature ($R^2 = 0.1087$, $P = 0.835$).

Mean size of diseased lobsters from the URI trawl survey (66.64 mm CL) was significantly larger than the mean size of non-diseased lobsters (64.3 mm CL; *t* test, df = 18827, $P = 0.001$) and size frequency distributions were significantly different (Kolmogorov-Smirnov, D = 0.1197; Fig. 3).

There was no relationship found between proportion soft and proportion infected ($R^2 = 0.0078$, $P = 0.32$). However, a pattern of decreasing proportion infected occurred over the molt season,

TABLE 3.

Proportion of lobsters with shell disease and total number sampled (in parentheses) in Wickford in Narragansett Bay and Whale Rock in Rhode Island Sound (URI trawl data). Significant values from the previous year are indicated with an asterisk.

	Whale Rock			Wickford			
	Males	Non-ovigerous females	Ovigerous females	Males	Non-ovigerous females	Ovigerous females	Weighted mean (z scores)
1995	0 (1,011)	0 (750)	0 (17)	0.0011 (918)	0.00195 (513)	0 (4)	5.82 E-06
1996	0.006 (1,725)	0.0033 (1,504)	0.0145 (69)	0.0043 (1,629)	0.0096 (935)	0 (2)	0.00551 (5.69)*
1997	0.02 (1,166)	0.014 (947)	0 (0)	0.0087 (1,031)	0.0022 (453)	0 (6)	0.01292 (3.5)*
1998	0.062 (598)	0.088 (506)	0.52 (41)	0.018 (1,555)	0.0235 (809)	0.33 (9)	0.0435 (7.8)*
1999	0.054 (78)	0.024 (84)	0.063 (17)	0.0198 (303)	0.019 (155)	0.11 (9)	0.0267 (2.32)*

TABLE 4.

Spearman's correlation matrix for variables tested in association with shell disease prevalence in the URI Trawl Survey. Variables include site, bottom temperature, legal or sublegal size, molt status, and cull status.

Spearman's rho	Disease	Site	Temperature	Sex	Size	Molt	Cull
Disease	1.00						
Site	0.037**	1.00					
	0.000						
Temperature	−0.062**	0.032**	1.00				
	0.000	0.000					
Sex	0.026**	0.120**	−0.034**	1.00			
	0.001	0.000	0.000				
Size	0.025**	−0.056**	−0.103**	−0.076**	1.00		
	0.001	0.000	0.000	0.000			
Molt	0.039**	0.016*	−0.024**	−0.016*	0.143**	1.00	
	0.000	0.027	0.001	0.027	0.000		
Cull	0.001	0.054**	−0.001	−0.006	−0.061**	0.069**	1.00
	0.859	0.000	0.845	0.457	0.000	0.000	

** Correlation is significant at the .01 level (two-tailed). * Correlation is significant at the .05 level (two-tailed). Listwise N. 18,291.

with an increasing trend in disease after the molt season that was consistent between sites. (Fig. 4).

Trap sampling in Dutch Harbor sites examined 2,909 lobsters. Shell disease showed a significant increase for each year between 1997 and 1999, reaching an infection rate of 19.11% (Fig. 2). The highest rates were seen in ovigerous females at 56.4% infected (Table 5).

Correlation analysis indicated positive relationships between disease and date, size and recapture status, and a negative relationship with molt and cull status and no correlation with sex or site (Table 6). A fourth order partial correlation controlling for date, molt status, cull, and recapture status showed a significant relationship between shell disease and size ($P < 0.001$), but not sex ($P = 0.071$). However, an evaluation of effect of sex on proportion infected using the Mann-Whitney test showed significant differences between males and non-ovigerous females ($z = -2.51$, $P = 0.012$), males and ovigerous females ($z = -7.72$, $P < 0.001$), and non-ovigerous females and ovigerous females ($z = -9.002$, $P < 0.001$).

Lobsters captured from the trap survey were significantly larger than those from the trawl survey (Kolmogorov-Smirnov, D = 0.236). Mean size of diseased lobsters (75.3 mm CL) was significantly larger than mean size of non-diseased lobsters (70.4 mm CL; t test, df = 3448, $P < 0.001$) and size frequency distributions were significantly different (Kolmogorov-Smirnov, D = 0.2476). There was a small peak in proportion infected at 50 mm CL, followed by an increasing trend from 70 to 100 mm CL (Fig. 3).

Low proportions of diseased lobsters were observed from April to July 1997, but increased over the summer and fall; 1998 had decreasing proportions from April to July when spawning and molting, increasing again in September and October (note: the large proportion of ovigerous females infected seen in 1998 may be due to small sample size for this sex category in that year; Fig. 5). The degree of disease coverage (SDI) was not independent of year sampled (chi-square = 146.8, $P < 0.001$); 1999 had increased proportions of SDI 1 and 2 (Table 7).

In the tag-recapture study 295 males were followed over the study period (Fig. 6). Of these, 19.7% became diseased. Of the total non-diseased lobsters, 25.3% molted and 74.7% did not. Thirty-five of them that were disease free at first capture were recaptured multiple times. Thirty-four of these developed the disease during the study; one molted and the disease did not reappear after 83 days. Twenty lobsters were diseased at first capture and followed with multiple recaptures. Once diseased, 81.8% of them did not molt. All but one of them worsened over time. Two mortalities (3.4%) were recorded for males with minor shell disease (SDI-1) and two mortalities were observed for non-diseased males (0.84%; Table 8).

One hundred thirty-eight non-ovigerous females were followed of which 10.9% of them became diseased (Fig. 7). Of those that were not observed to contract the disease, 42.6% molted, while 57.4% did not. One mortality was observed (0.72%). Seventeen lobsters were recaptured multiple times and all developed the disease. Before acquiring the disease, 73% of them did not molt and 27% did molt. After acquiring the disease, 36.4% of them molted and did not show signs of the disease after a mean of 88.3 days. Forty-four females became ovigerous during the study period and 25% of them became infected. Of those that were not diseased, 9.1% molted and lost eggs. Of the ovigerous diseased females, 27.3% of them molted and/or lost eggs (Table 9).

Shell disease outcome was independent of size (chi-square, df = 2, $P < 0.058$), season (chi-square = 2.24, df = 3, $P = 0.524$),

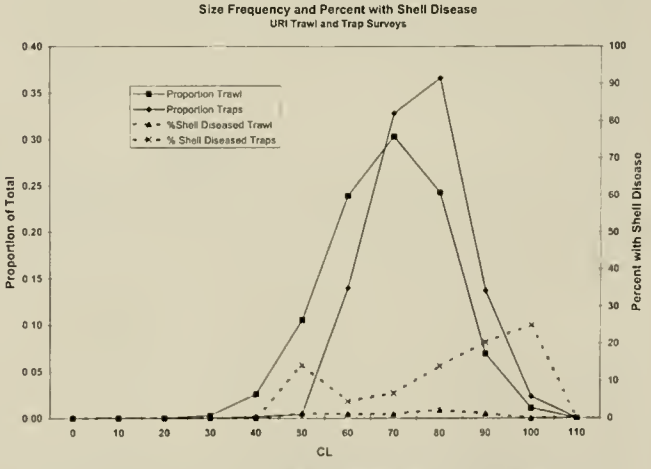

Figure 3. Size frequency of shell diseased and non-diseased lobster from URI trawl survey and trap survey from Dutch Harbor.

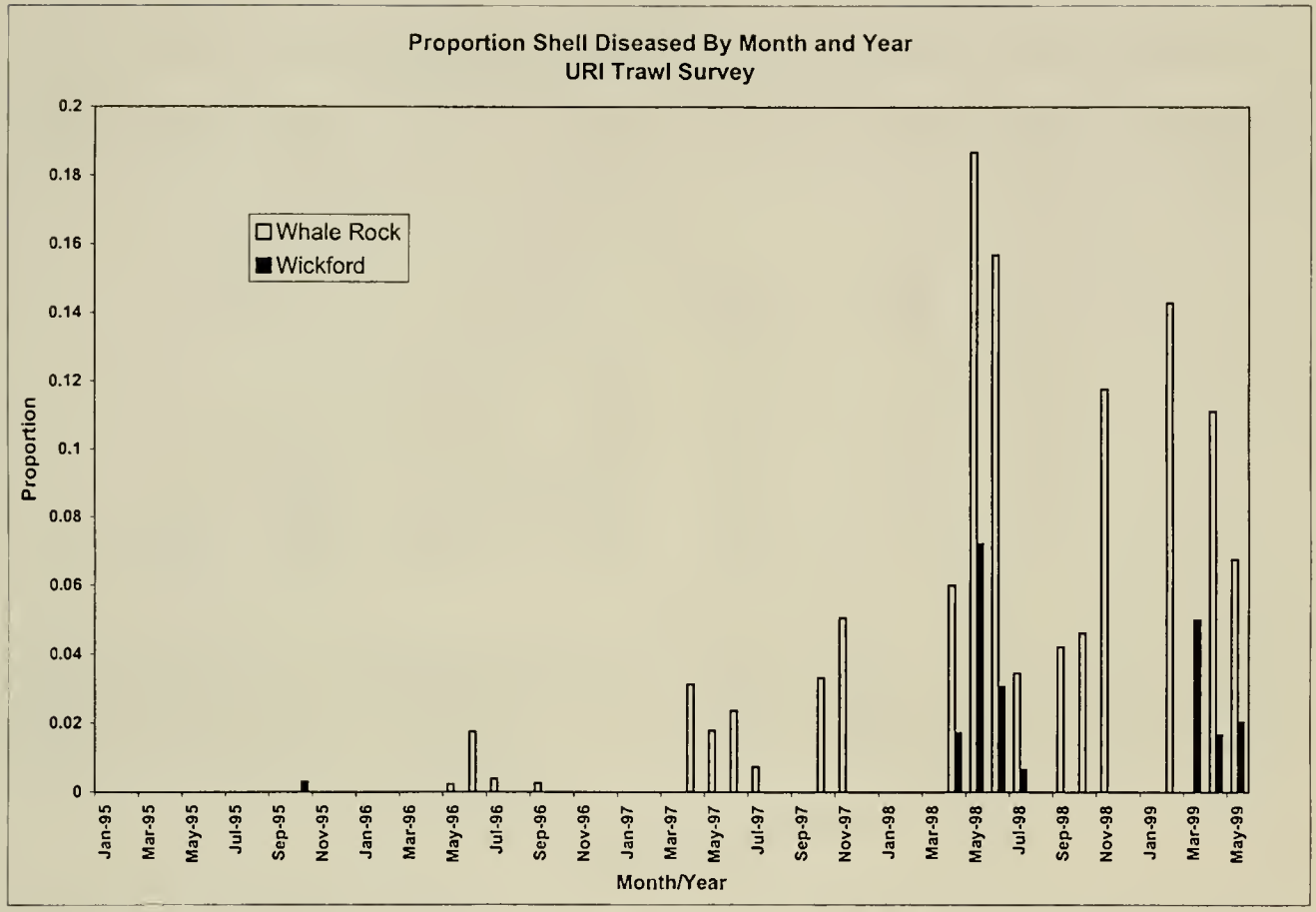

Figure 4. Proportion of lobsters with shell disease by month and year from the URI trawl survey. Molt usually occurs from April to June and August to October of each year. Sampling occurs year round.

and sex (chi-square = 5.095, df = 4, $P = 0.278$) (Fig. 8). No relationship was found between days between recaptures and shell disease difference; in some cases the greatest increase in infection occurred in short recapture periods. By examining short recapture intervals (less than 36 days) the first appearance of shell disease was estimated (from 0–1 SDI). These were confined to September through November in 1997 ($n = 11$); June, September and October in 1998 ($n = 6$); and August through October in 1999 ($n = 10$). Worsening of shell disease condition was rapid between September and October.

DISCUSSION

The routine surveys described here opportunistically recorded a shell disease episode affecting the inshore Rhode Island lobster population. Beginning in 1995, there were low proportions of minor shell diseased lobsters observed in the URI trawl survey; however, by 1999, a greater percentage of lobsters were showing evidence of infection in all surveys, with ovigerous females being the most affected. Each survey revealed slightly different trends and/or proportions observed. The prevalence of shell disease observed in 1997 and 1999 from the DEM survey coincides with that observed in Dutch Harbor. The URI trawl survey reported much lower overall proportions than other surveys.

The differing sampling designs and gear types probably account for the majority of the variation observed, although no information is available regarding changes in lobster behavior (i.e. catchability) as a result of disease. The large sample size examined from the DEM survey may increase observation error and may not represent the population as a whole because catchability in the

TABLE 5.

Proportion of lobsters with shell disease and total numbers sampled (in parentheses) in Dutch Harbor (URI trap survey). Significant differences from the previous year are indicated with an asterisk.

Dutch Harbor	Males	Non-ovigerous females	Ovigerous	Weighted mean (z scores)
1997	0.0599 (901)	0.0298 (436)	0.196 (56)	0.056
1998	0.099 (383)	0.053 (225)	0.625 (8)	0.08903 (2.54*)
1999	0.1625 (449)	0.186 (339)	0.564 (39)	0.1911 (5.71*)

TABLE 6.

Spearman's correlation matrix for variables tested in association with shell disease infection in Dutch Harbor. Variables include site, sex, size, molt status, cull status, date, and recapture status.

Spearman's rho	Disease	Site	Sex	Size	Molt	Cull	Date	Recap
Disease	1.00							
Site	0.010	1.00						
	0.547							
Sex	0.019	0.116**	1.00					
	0.277	0.000						
Size	0.164**	−0.055**	−0.085**	1.00				
	0.000	0.001	0.000					
Molt	−0.067**	0.011	−0.022	−0.114**	1.00			
	0.000	0.518	0.194	0.000				
Cull	−0.060**	−0.050**	0.006	−0.061**	−0.001	1.00		
	0.000	0.004	0.742	0.000	0.954			
Date	0.206**	−0.067**	0.073**	0.164**	−0.230**	−0.115**	1.00	
	0.000	0.000	0.000	0.000	0.000	0.000		
Recap	0.069**	−0.068**	−0.006	0.214**	−0.084**	−0.004	0.100*	1.00
	0.000	0.000	0.726	0.000	0.000	0.811	0.000	

Listwise N, 3,388. ** Correlation significant at the 0.01 level (two-tailed).

traps increases shortly after the molt. If the proportions infected are at their lowest value at the time of highest catchability, the infected estimate may be low. The size frequency may also be different than those captured in the other two surveys because of escape vents. However, the DEM survey covers a larger area of the inshore waters and may be more representative of the whole. Many differences between the URI trawl survey and other surveys have been noted (ASMFC 2000), especially in reference to trends and

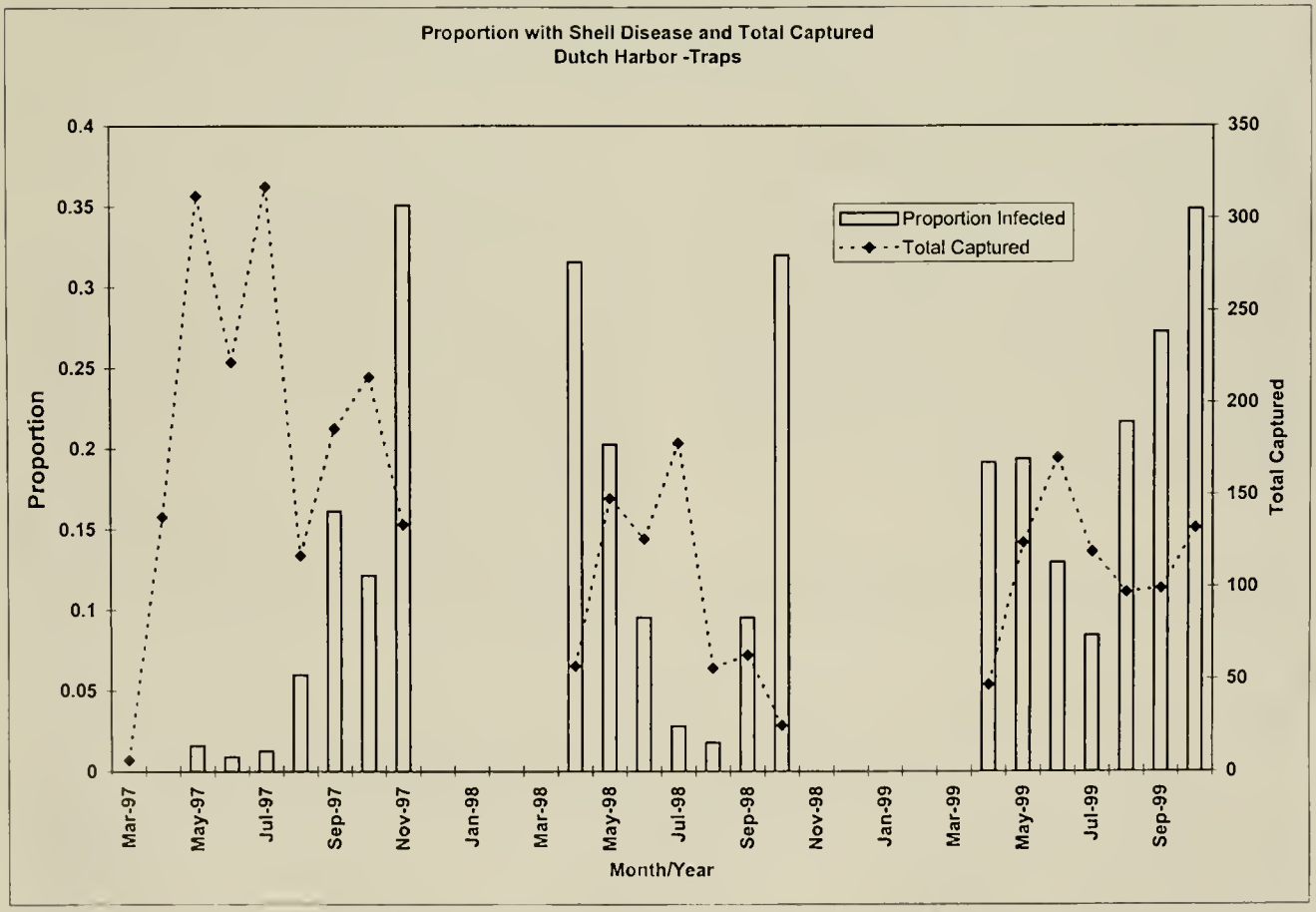

Figure 5. Proportion of lobsters with shell disease by month/year from Dutch Harbor trap survey. Molting usually occurs from April to June and August to October each year. Note: no sampling occurs from December 1997 to March 1998 and November 1998 to March 1999.

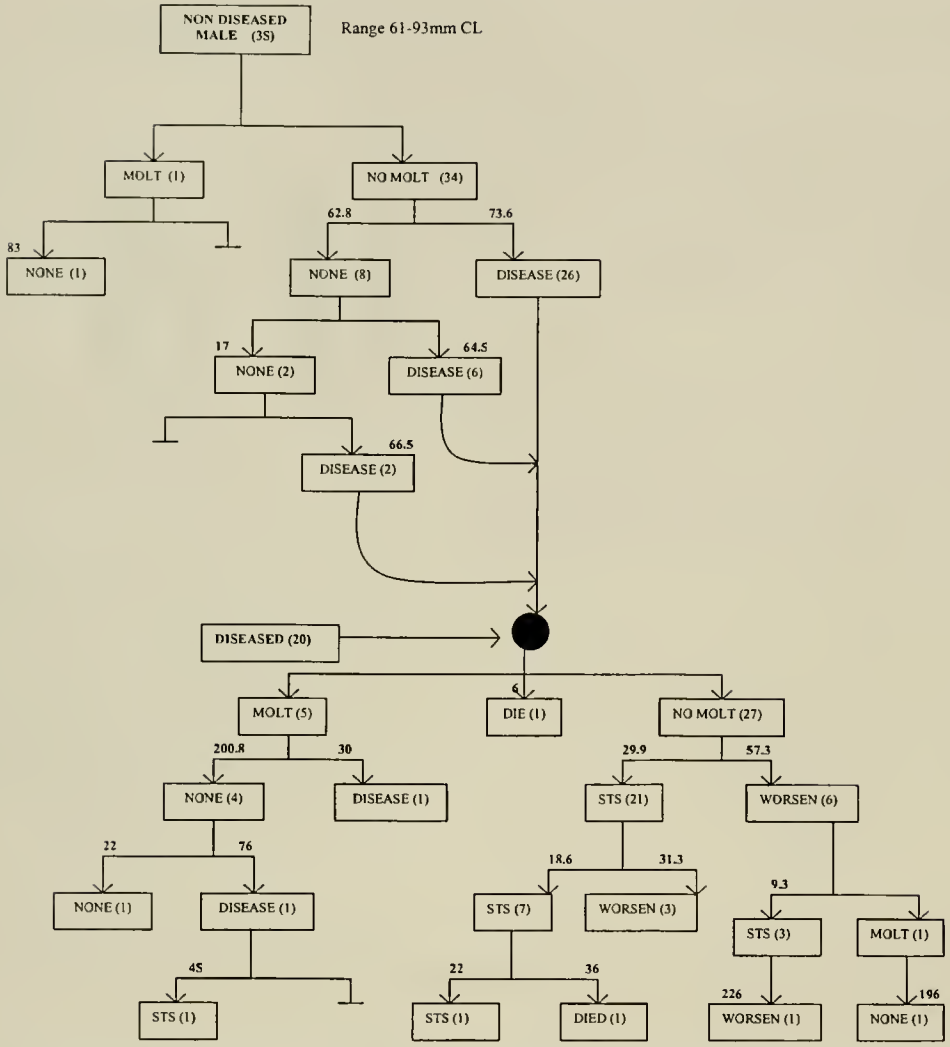

Figure 6. Pathways followed by shell diseased males followed during tag-recapture study. Numbers inside boxes corresponds to numbers of individuals observed; numbers in bold indicate mean days between recaptures, unless only one individual is represented. STS, stays the same; None, no disease observed.

numbers. This may be a consequence of the fixed stations, the weekly sampling, and/or the habitat type. The URI trawl may be capturing migrating lobsters since it occurs over soft featureless bottom consisting of mud/sand substrate. The trawl captures a larger range of size classes. The incomplete year of sampling in the URI trawl survey in 1999 most likely is responsible for the decrease seen that year, especially since there was an early molt. Dutch Harbor represents only one area and may have unusually high or low infection rates. The lobster reef site is in a voluntary no-fishing zone and proportionally larger lobsters are captured there than at other sites. These have a longer time to develop shell disease than those removed by the fishery and may present a more accurate size-related effect of shell disease. The higher rates could also be related to habitat type as well. Since many of these lobsters are handled through multiple recaptures, stress may influence the progression of shell disease. Tagging and handling may affect molting and reproduction as well. Oocyte resorption has been as-

TABLE 7.

Proportion of lobsters (sexes combined) with shell disease index (SDI) 0 to 4 in years 1997 through 1999 from Dutch Harbor.

	1997	1998	1999
SDI-0	0.943	0.911	0.808
SDI-1	0.032	0.047	0.138
SDI-2	0.007	0.013	0.041
SDI-3	0.009	0.008	0.002
SDI-4	0.009	0.021	0.011

TABLE 8.

Proportion of male lobsters following designated pathways.

Lobster types	Molt	No molt	Die
Non-diseased males	0.253	0.747	0.0084
Diseased males[1]	0.03	0.97	0
Diseased males[2]	0.28	0.818	0.034

[1] Males that entered pathway non-diseased.
[2] Males that entered pathway diseased.

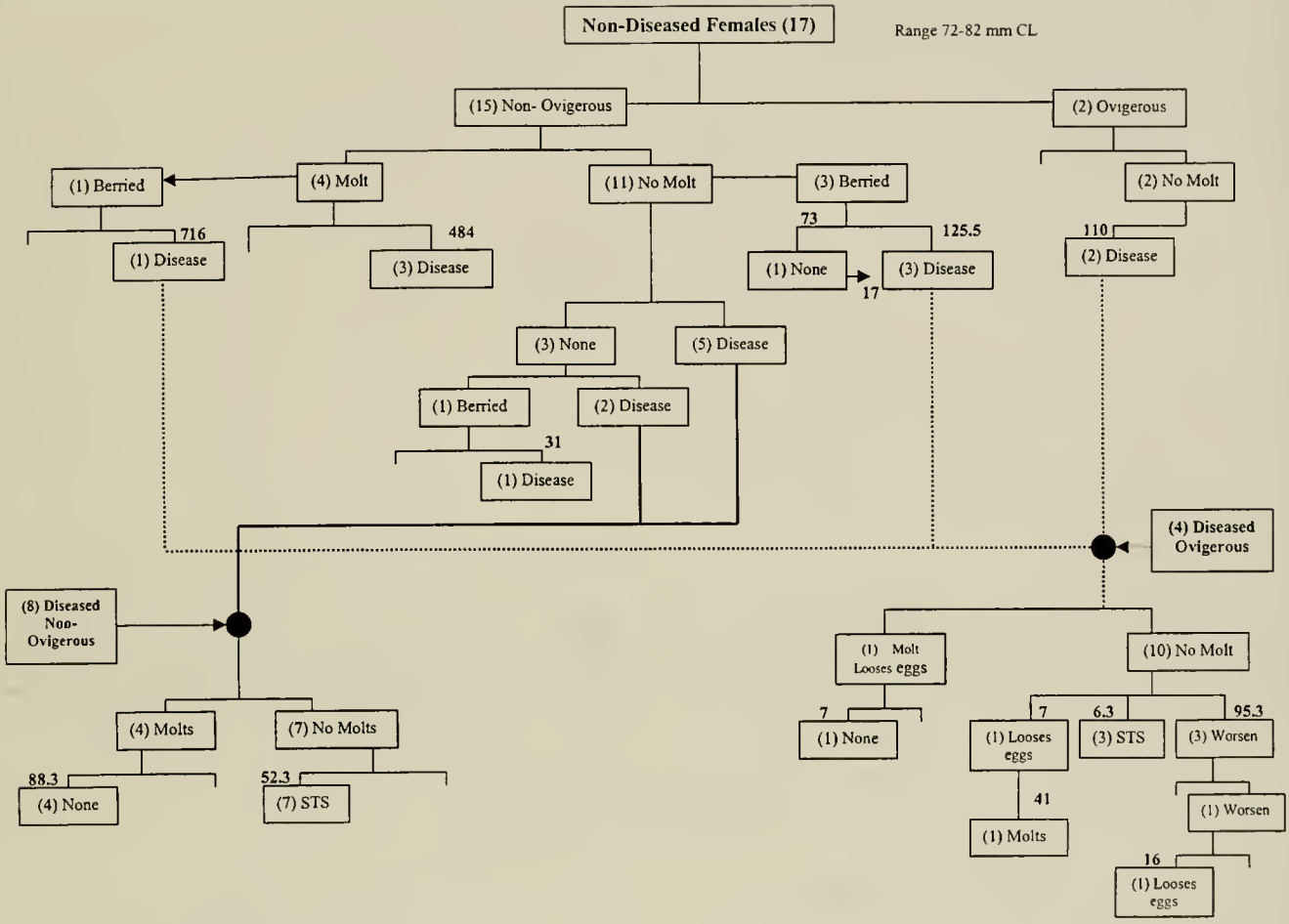

Figure 7. Pathways followed by shell diseased females followed during tag-recapture study. Numbers inside boxes corresponds to numbers of individuals observed; numbers in bold indicate mean days between recaptures, unless only one individual is represented. STS, stays the same; None, no disease observed; Berried, egg-bearing.

sociated with stressful environmental conditions (Waddy et al. 1995). Cooper (1970) reported a delayed molt or reduced molt increment under certain circumstances as a result of tagging.

Sampling time had a large impact on observed proportion infected, with pre-molting peaks seen in both URI sites in the spring. The peak after the second molt period in Whale Rock corresponded to the time period in which infection rates also rose in Dutch Harbor. Malloy (1978) observed that more disease was established in lobsters in post-ecdysis than in pre-ecdysis, which could explain the higher proportions observed in the fall. This may be the more serious indication of problems in the population since those lobsters must overwinter with the degraded shell condition. This may result in a weakened state that makes these lobsters more vulnerable to other pathogens, predators, or adverse environmental conditions (Smolowitz et al. 1992). There were verbal accounts of lobsters being consumed by starfish in traps over the winter fishery. It is unknown if healthy lobsters would be susceptible to attacks by starfish, even when they are lethargic because of low water temperatures.

There was evidence from this study that the proportion infected increased with size, especially in the no-fishing area where larger lobsters were present. This supports the hypothesis that molting

TABLE 9.

Proportion of female lobsters following designated pathways.

Lobster types	Molt	No molt	Die
Non-diseased/non-ovigerous females	0.426	0.574	0.0072
Diseased/non-ovigerous females[1]	0.27	0.73	0
Diseased/non-ovigerous females[2]	0.364	0.636	0
Non-diseased/ovigerous females	0.091	0.909	0
Diseased/ovigerous females	0.273	0.727	0

[1] Females that entered pathway non-diseased.
[2] Females that entered pathway diseased.

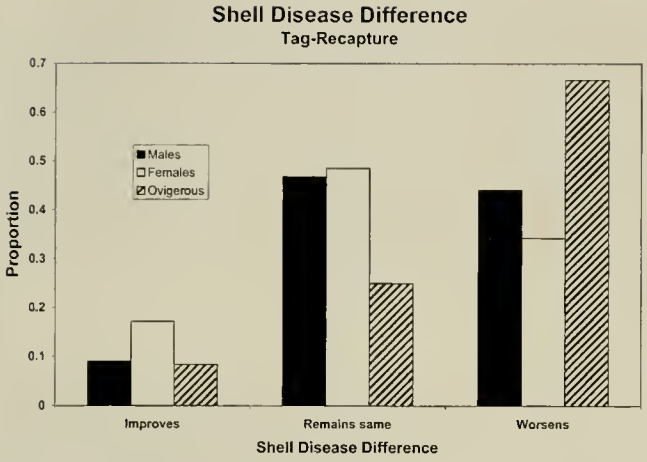

Figure 8. Proportion of shell diseased lobsters by sex that improve, remain the same, or worsen during tag-recapture events.

frequency affects proportion diseased. However, there were considerable numbers of smaller lobsters infected as well. The molt may physically remove signs of shell disease, however, the degree of damage to the new shell may be related to when the diseases occurs in the molt cycle and how quickly it progresses through the shell layers. If the disease is the result of an internal metabolic disturbance, molting might not provide extended respite from infection. Many of the lobsters followed in the tag-recapture study redeveloped shell disease after molting. Field observations of newly molted lobsters revealed many with darkened areas.

There was no relationship found in this study between bottom temperature and proportion of shell diseased lobsters. This was not surprising since shell disease occurs worldwide in crustaceans from all climatic conditions ranging from ice-covered lakes to semi-tropical estuaries and deep water (Rosen 1970). Malloy (1978) reported more disease occurred in lobsters held in water temperatures of 2 °C to 5 ° rather than at higher temperatures, which would imply that the disease does not recess during the winter months, but continues to progress. Hood and Meyers (1974) found the highest populations of chitinoclastic bacteria in the environment occurred during spring and early summer when median temperatures were above 16.9 °C, but shell disease in blue crabs was more prevalent during late fall and winter.

Lobsters fed an insufficient diet may also be more vulnerable to shell disease (Malloy 1978). Dietary deficiencies were a factor in the development of shell disease in juvenile American lobsters (4th through12th stage) because of problems in epicuticular repair (Fisher et al. 1976). Prince et al. (1995) reduced shell disease incidence in pounds by feeding pellets containing a higher protein and crude fat content.

Cook and Lofton (1973) found that in blue crabs, only mechanically damaged areas were susceptible to shell disease. Mechanical injuries caused by handling, ecdysis, aggressiveness, and high stocking densities have been blamed for shell disease in prawns (Delves-Broughton and Poupard 1976). Johnson (1983) reported that chitinoclastic bacteria did not cause harm in natural unstressed environments. Getchell (1989) reported that only in degraded or crowded conditions does shell disease appear to be highly contagious. Other degraded conditions may include exposure to sewage, sludge, heavy metals, or dredge spoils. Healthy lobsters held in aerated seawater with sewage for up to 6 wk developed shell disease, whereas control lobsters held in clean water did not (reported in Stewart 1980). However, no field study to date has been able to effectively demonstrate a direct cause and effect relationship (Young and Pearce 1975, Estrella 1984, Ziskowski et al. 1996).

Several of these factors may play a role in the observed increased lobster infection rates. It appears to be occurring on a large geographic scale, which would point to a common environmentally induced cause. A similar hypothesis has been proposed for the recent increases in abundance reported for recruit sized lobsters in South of Cape Cod Long Island Sound Stock (SCCLIS) assessment area (ASMFC 2000). These very high abundance estimates may increase densities, creating more opportunities for aggressive encounters because of food or space limitations or stressed conditions leading to metabolic dysfunction or contagious conditions. However, few of the observed shell-diseased lobsters displayed obvious physical damage; juvenile and adult lobsters are not thought to be habitat limited (Wahle and Steneck 1992) and since lobsters are opportunistic feeders, it is unlikely that food supply is limiting. The observed shell disease etiology fits the description provided by Bullis et al. (1988) for the endogenous origin of shell disease which may be a consequence of immunosuppression or of failure of metabolic processes associated with shell repair and maintenance. The etiology and mechanism for this are not obvious.

Possible consequences for the population are numerous. Taylor (1948) found a 71% mortality associated with shell-diseased lobsters in a laboratory setting compared with 6% in control lobsters. Large lesions may cause mortality during ecdysis if there is adhesion between the exoskeleton and underlying tissues (Martin and Hose 1995). Winter mortalities due to shell disease have historically been recorded in Nova Scotian pounds (Hess 1937, Taylor 1948, Malloy 1978, Getchell 1989). Mortality rates attributed to shell disease during pounding were reported to be 6.5% (Prince et al. 1995). Secondary mortality effects due to predation or parasites are unknown.

It was surprising to find that three diseased and one non-diseased ovigerous female lobsters who molted late in the season shed their eggs. Several area fishermen have also reported finding cast shells with eggs attached in their traps. However, the eggs have not been shown to be fertilized or even viable. It is known that some females will reabsorb the yolk from mature oocytes (Waddy et al. 1995), which may be related to warm winter temperatures. However, it is rare for lobsters to molt while carrying eggs (Waddy and Aiken 1991). Usually the molting and spawning cycles are synchronized by temperature and are under endocrine control, and molting is believed to take precedence over reproduction (Waddy et al. 1995). The consequences for egg production may be severe, regardless of the ultimate pathway followed. Since the ovigerous females are displaying the highest rate of infection and if they experience the extreme mortality rates as indicated by Taylor (1948) then our reproducing lobsters may suffer higher mortality rates if they continue to brood their eggs. If they molt, then the potential egg production is lost and they may recontract the disease and legal-sized females then become vulnerable to the fishery.

This is the first study that has been conducted that examines the effects of shell disease on lobsters in the field. There is a great need for future work, especially concerning site differences, mortality rates, and biological and economic consequences to the population and the fishery. With the example of the recent devastating mortality event in Western Long Island Sound lobsters, we need to

gain an understanding of the mechanisms and causes of disease outbreak in our valuable marine crustaceans.

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BRACHYURAN COMMUNITY IN UBATUBA BAY, NORTHERN COAST OF SÃO PAULO STATE, BRAZIL

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ABSTRACT A brachyuran crab assemblage from eight transects at a non-consolidated sublittoral site in Ubatuba Bay was studied on a monthly basis from September 1995 to August 1996. Data about number of individuals of 50 species found and other information such as distribution of the dominant crabs are reported. The family Majidae was represented by 13 species, followed by Xanthidae (13), Portunidae (10), Leucosiidae (5), Calappidae (2), Dromiidae (2), Parthenopidae (2), Goneplacidae (1), Pinnotheridae (1), and Ocypodidae (1). The brachyuran taxocoenosis was dominated by *Callinectes ornatus* (60.4%), *Callinectes danae* (18.8%), and *Hepatus pudibundus* (7.7%), representing together 86.9% of the total number of collected brachyurans. The Shannon-Weaver diversity index ranged from 1.10 to 2.06 between transects, and from 1.34 to 2.22 between months, depending more on equitability than on richness.

KEY WORDS: Biodiversity, Brachyura, crabs, Crustacea, Ubatuba, Brazil

INTRODUCTION

With approximately 5,000 species described worldwide, the Brachyura is a highly significant group of marine crustaceans playing an important role in the marine trophic chain (Melo 1996). Recently our group listed 315 brachyuran species living along the Brazilian coast (Pohle et al. 1999) and this number continues to be modified both by new species descriptions and new records of exotic brachyurans.

The South Atlantic coastal zone can be functionally divided into two regions: the subtropical Brazilian coast (from 22°S to 33°S) of about 3,000 km, and the Patagonian region consisting of the temperate coasts of Southern Brazil, Uruguay and Northern Argentina (from 33°S to 42°S) extending over 2,900 km. Located along the northern coastline of the State of São Paulo, the Ubatuba region is an important area for crustacean investigations, particularly on Brachyura. This region comprises the coastal portion of the Biogeographic Province of the State of São Paulo, a zone of faunal transition (Palácio 1982). The area as a whole possesses a mixture of faunas of both tropical and Patagonian origin (Sumida and Pires-Vanin 1997). In addition, Ubatuba Bay is its fairly pristine and is used as a standard for comparison with other marine habitats strongly influenced by man (Mantelatto and Fransozo 1999a). For this reason in recent years there has been an impressive number of studies of the intertidal zone and continental shelf centered on the crab faunal composition of a variety of habitats in the Ubatuba area. Forneris (1969) performed the pioneering study which provided a brachyuran check-list in Flamengo Bay; Abreu (1980) described ecological aspects in an estuarine area of Ubatuba; Pires (1992) reported the structure and dynamics on the continental shelf offshore of Ubatuba; Fransozo et al. (1992) established the composition and the distribution at the non-consolidated sublittoral from Fortaleza Bay; Hebling et al. (1994) reported the crabs sampled in the Anchieta Island region; Mantelatto and Corrêa (1996) described the composition and seasonal variations of the species living on the algae *Sargassum cymosum* C. Agarth, 1820 from three different Ubatuba beaches; Pinheiro et al. (1997) studied the composition and the relative abundance of crabs associated with sand reefs created by *Phragmatopoma lapidosa* Kimberg, 1867, and Mantelatto and Souza-Carey (1998) reported the species inhabiting the bryozoan colonies of *Schyzoporella unicornis* (Johnston 1847). The purpose of the present paper is to report the species composition of brachyuran crabs from a sublittoral location with non-consolidated sediments in Ubatuba Bay as a contribution to the study of the biodiversity of Brachyura from the São Paulo coast.

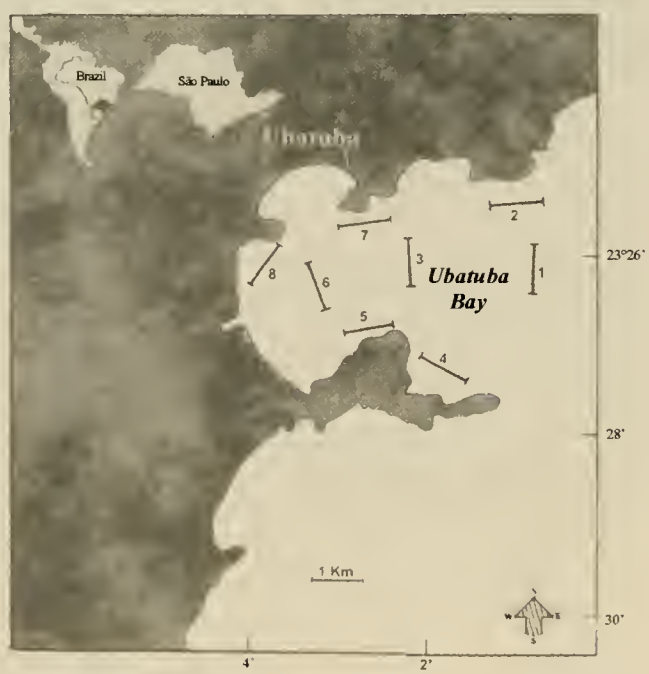

Figure 1. Map of Ubatuba Bay (São Paulo State) showing the position of the sampling transects.

TABLE I.

Total species composition and number of individuals in each transect in Ubatuba Bay calculated for whole year. (CN, constancy; Co, constant; Ac, accessory, and Ad, accidental).

Family/Species	Transects									
	I	II	III	IV	V	VI	VII	VIII	TOTAL	CN
DROMIIDAE										
Cryptodromiopsis antillensis (Stimpson 1858)	–	–	–	3	8	–	–	–	11	Co
Hypoconcha arcuata (Stimpson 1858)	–	–	–	–	2	–	–	–	2	Ad
CALAPPIDAE										
Hepatus pudibundus (Herbst 1785)	266	50	86	96	35	368	70	15	986	Co
Calappa gallus (Herbst 1803)	–	–	–	5	2	–	–	–	7	Ac
LEUCOSIIDAE										
Lithadia brasiliensis (von Martens 1872)	–	–	–	–	1	–	–	–	1	Ad
Persephona crinita (Rathbun 1931)	–	–	1	–	–	2	2	–	5	Ad
Persephona lichtensteinii (Leach 1817)	–	–	4	–	–	14	6	1	25	Co
Persephona mediterranea (Herbst 1794)	105	2	1	12	2	3	1	–	126	Co
Persephona punctata (Linnaeus 1758)	20	5	5	70	68	181	11	–	360	Co
MAJIDAE										
Apiomithrax violaceus (A. Milne Edwards 1868)	–	–	–	–	9	1	–	–	10	Ac
Collodes inermis (A. Milne Edwards 1878)	–	–	–	–	4	–	–	–	4	Ad
Collades rostratus (A. Milne Edwards 1878)	–	–	–	1	–	–	–	–	1	Ad
Libinia ferreirae (Brito Capello 1871)	5	7	5	12	5	9	7	–	50	Co
Libinia spinosa (H. Milne-Edwards 1834)	7	1	3	1	–	3	1	–	16	Ac
Microphrys bicornutus (Latreille 1825)	1	–	1	–	–	–	–	–	2	Ad
Notolopas brasiliensis (Miers 1886)	–	–	–	–	6	–	–	–	6	Ac
Pelia rotunda (A. Milne Edwards 1875)	–	3	2	1	–	–	–	–	6	Ac
Pitho lherminieri (Schramm 1867)	–	–	–	1	–	–	–	–	1	Ad
Podachela gracilipes (Stimpson 1871)	–	–	–	–	1	–	–	–	1	Ad
Podochlae riisei (Stimpson 1860)	–	–	–	1	–	–	–	–	1	Ad
Pyromaia tuberculata (Lockington 1876)	–	–	–	–	1	–	–	–	1	Ad
Stenorhynchus seticornis (Herbst 1788)	–	–	–	–	11	–	–	–	11	Ac
PARTHENOPIDAE										
Parthenope (*Parthenope*) *agona* (Stimpson 1871)	–	–	–	–	1	–	–	–	1	Ad
Parthenope (*Platylambrus*) *guerini* (B. Capello 1871)	–	–	–	–	1	–	–	–	1	Ad
PORTUNIDAE										
Arenaeus cribrarius (Lamarck 1818)	213	98	2	1	–	–	85	47	446	Co
Callinectes danae (Smith 1869)	4	16	86	9	–	393	272	1,626	2,406	Co
Callinectes ornatus (Ordway 1863)	436	422	489	1,111	834	2,910	657	866	7,725	Co
Callinectes sapidus (Rathbun 1896)	–	–	–	–	–	2	1	1	4	Ac
Charybdis hellerii (A. Milne Edwards 1867)	–	1	–	4	131	3	–	–	139	Co
Cronius ruber (Lamarck 1818)	–	–	–	1	1	–	–	–	2	Ad
Portunus ordwayi (Stimpson 1860)	–	–	–	–	7	–	–	–	7	Ad
Portunus spinicarpus (Stimpson 1871)	–	4	–	–	10	–	–	–	14	Ad
Portunus spinimanus (Latreille 1819)	–	1	–	65	143	5	1	–	215	Co
Portunus ventralis (A. Milne Edwards 1879)	–	–	–	–	1	–	–	–	1	Ad
XANTHIDAE										
Eurypanopeus abbreviatus (Stimpson 1860)	–	–	–	–	–	1	–	–	1	Ad
Hexapanopeus sp.	–	–	–	–	–	1	–	–	1	Ad
Hexapanopeus paulensis (Rathbun 1930)	–	4	1	9	21	15	–	–	50	Co
Hexapanopeus schmitti (Rathbun 1930)	–	1	–	24	35	19	–	–	79	Ac
Menippe nodifrons (Stimpson 1859)	–	–	–	–	1	1	1	–	3	Ad
Micropanope nuttingi (Rathbun 1898)	–	–	–	1	–	–	–	–	1	Ad
Panopeus americanus (Saussure 1857)	–	–	–	2	1	–	–	–	3	Ad
Panopeus bermudensis (Benedict and Rathbun 1981)	–	–	1	–	–	–	–	–	1	Ad
Pilumnoides hassleri (A. Milne Edwards 1880)	–	–	–	4	2	–	1	–	7	Ad
Pilumnus diomedeae (Rathbun 1894)	–	–	–	–	1	–	–	–	1	Ad
Pilumnus reticulatus (Stimpson 1860)	–	–	–	–	20	2	–	–	22	Co
Pilumnus spinosissimus (Rathbun 1898)	–	–	–	1	–	–	–	–	1	Ad
Xanthidae sp.	–	–	–	2	–	–	–	–	2	Ad
GONEPLACIDAE										
Eucratopsis crassimanus (Dana 1852)	–	–	–	13	7	1	–	–	21	Ac
PINNOTHERIDAE										
Pinnixa sp.	–	–	1	–	–	–	1	–	2	Ad
OCYPODIDAE										
Ucides cordatus (Linnaeus 1763)	–	–	–	–	–	–	–	1	1	Ad
TOTAL	1,057	615	688	1,450	1,372	3,934	1,117	2,557	12,790	
Number of species	9	14	15	25	31	20	15	7		

TABLE 2.

Total number of individuals per month for all eight subareas combined, collected from September 1995 (S) to August 1996 (A).

Family/Species	Months												Total
	S	O	N	D	J	F	M	A	M	J	J	A	
DROMIIDAE													
Cryptodromiopsis antillensis	–	1	1	–	1	3	1	1	–	–	–	3	11
Hypoconcha arcuata	2	–	–	–	–	–	–	–	–	–	–	–	2
CALAPPIDAE													
Hepatus pudibundus	133	101	117	40	88	84	55	39	29	53	130	117	986
Calappa gallus	1	–	1	1	–	–	–	–	–	2	2	–	7
LEUCOSIIDAE													
Lithadia brasiliensis	1	–	–	–	–	–	–	–	–	–	–	–	1
Persephona crinita	–	–	–	1	–	–	–	–	–	–	2	2	5
Persephona lichtensteinii	6	5	–	–	3	–	1	1	1	–	6	2	25
Persephona mediterranea	21	11	18	3	15	3	9	–	1	3	29	13	126
Persephona punctata	70	17	43	6	9	11	6	12	10	8	54	114	360
MAJIDAE													
Apiomithrax violaceus	1	1	1	–	–	–	4	3	–	–	–	–	10
Collodes inermis	–	1	–	–	–	–	–	–	1	–	–	2	4
Collodes robustus	–	1	–	–	–	–	–	–	–	–	–	–	1
Libinia ferreirae	2	4	11	2	–	2	–	2	1	1	14	11	50
Libinia spinosa	–	–	4	–	1	–	–	–	6	–	4	1	16
Microphrys bicornutus	–	1	–	1	–	–	–	–	–	–	–	–	2
Notolopas brasiliensis	–	1	–	–	1	–	2	–	–	–	1	1	6
Pelia rotunda	–	1	1	–	–	–	–	1	–	–	1	2	6
Phito lherminieri	–	–	–	–	–	–	1	–	–	–	–	–	1
Podochela gracilipes	–	–	–	–	–	–	1	–	–	–	–	–	1
Podochela riisei	–	–	–	–	–	–	–	–	–	–	–	1	1
Pyromaia tuberculata	1	–	–	–	–	–	–	–	–	–	–	–	1
Stenorhynchus seticornis	–	–	4	–	–	–	1	1	–	–	2	3	11
PARTHENOPIDAE													
Parthenope agona	–	–	–	–	–	–	1	–	–	–	–	–	1
Parthenope (Platylambrus) guerini	–	–	–	–	–	–	–	–	1	–	–	–	1
PORTUNIDAE													
Arenaeus cribrarius	33	33	28	20	34	66	35	27	39	45	61	25	446
Callinectes danae	104	47	82	28	114	515	542	234	162	269	189	120	2406
Callinectes ornatus	824	426	592	466	696	1031	692	662	430	466	648	792	7725
Callinectes sapidus	1	1	–	–	1	1	–	–	–	–	–	–	4
Charybdis hellerii	7	1	7	–	–	–	16	40	32	2	15	19	139
Cronius ruber	–	–	–	–	–	–	–	2	–	–	–	–	2
Portunus ordwayi	–	–	–	–	–	–	–	7	–	–	–	–	7
Portunus spinicarpus	–	–	–	5	–	9	–	–	–	–	–	–	14
Portunus spinimanus	61	15	35	6	15	5	2	23	21	11	8	13	215
Portunus ventralis	–	–	–	–	–	–	–	–	1	–	–	–	1
XANTHIDAE													
Eurypanopeus abbreviatus	–	–	–	1	–	–	–	–	–	–	–	–	1
Hexapanopeus sp.	–	–	–	–	1	–	–	–	–	–	–	–	1
Hexapanopeus paulensis	1	–	–	3	7	3	4	18	5	2	2	5	50
Hexapanopeus schmitti	1	–	–	3	9	5	47	14	–	–	–	–	79
Menippe nodifrons	–	–	–	–	–	1	1	1	–	–	–	–	3
Micropanope nuttingi	–	–	–	–	–	–	–	1	–	–	–	–	1
Panopeus americanus	1	–	–	–	–	2	–	–	–	–	–	–	3
Panopeus bermudensis	–	–	–	–	–	–	–	1	–	–	–	–	1
Pilumnoides hassleri	1	1	–	–	5	–	–	–	–	–	–	–	7
Pilumnus diomedeae	–	–	–	–	1	–	–	–	–	–	–	–	1
Pilumnus reticulatus	–	1	4	1	–	–	12	2	–	–	1	1	22
Pilumnus spinosissimus	–	–	–	–	–	–	–	–	–	–	1	–	1
Xanthidae sp.	–	–	–	–	–	–	–	–	–	–	2	–	2
GONEPLACIDAE													
Eucratopsis crassimanus	2	–	–	–	4	9	2	3	–	–	1	–	21
PINNOTHERIDAE													
Pinnixa sp.	–	–	–	1	1	–	–	–	–	–	–	–	2
OCYPODIDAE													
Ucides cordatus	–	–	–	–	–	–	–	–	–	–	1	–	1
Number of species	21	20	16	17	19	16	21	22	15	11	22	20	

MATERIALS AND METHODS

Ubatuba Bay (23°26′S and 45°02′W) is adjacent to the town of Ubatuba situated on the northern coast of São Paulo, Brazil. The area of the bay is about 8 km^2 with a width of approximately 4.5 km at the entrance.

The study site was divided into eight subareas selected for their relation to the bay mouth, the presence of a rocky wall or a beach along the boundaries, the inflow of fresh water, the proximity of offshore water, depth, and granulometric composition. Each transect was assigned to a subarea for sampling of crabs and measurement of environmental factors (Fig. 1). During the study the environmental data was sampled throughout full transect and was checked at the beginning, middle, and end of each transect sample. There was no change in this data throughout each transect. Depth ranged from 2.5 (subarea 2) to 18.5 m (subarea 1), temperature ranged from 19.2 °C to 20.1 °C, salinity ranged from 33.5‰ to 34.8‰, and dissolved oxygen ranged from 5.21 to 5.87 mg/L. The overall organic matter content in bottom sediments ranged from 2.0% (subarea 2) to 30.2% (subarea 5) and fine sediments (<0.250 mm) prevailed in most subareas. Water samples were collected from the bottom using a Nansen bottle. Temperature was measured with a thermometer attached to the bottle, salinity was measured using an optical refractometer (Atago S/1000), and dissolved oxygen was measured by the Winckler method modified by the addition of azide. Depth was measured in each sampling station using a graduated rope that was attached to the Van-Veen grab sampler (1/40 m^2) used for sampling sediment. Sediment (≈ 200 g) was dried at 70 °C for 72 h before organic matter and grain size analyses. The Wenthworth (1922) scale was used for the grain size analyses. The phi (Φ = mean diameter) value was used according to Suguio (1973) to calculate the central sediment tendency. Organic matter was obtained by ash-weighting, three aliquots of 10 g each per subarea per month were heated in porcelain crucibles for 3 h at 500 °C and then reweighed. Detailed descriptions of physical and chemical features characterizing this area and statistical similarity of environmental factors among transects can be found in Mantelatto and Fransozo (1999a).

Sampling of crabs occurred monthly from September 1995 to August 1996. The sample was performed at a diurnal 1-km-long trawl transect at each of the eight sampling subareas during a three consecutive days per month. The catches of crabs were done by trawler equipped with double rigged nets (3.5-m wide mouth, 10 mm of mesh size cod end). Each trawl was performed with velocity and time adequate to prevent significant scape from each net in function of the differences on bottom substrate surface and tidal currents. Immediately after capture all crabs were placed on ice and frozen until being examined in the laboratory.

The Constancy Index (C) for each species was calculated according to Dajoz (1983): $C = P \times 100/P$, where "P" is the number of samples in which a given species was recorded, and "P" is the total number of samples analyzed. Species were then classified into three different constancy categories; *i.e.* constant ($C \geq 50$ %), accessory ($25\% < C < 50\%$), and accidental ($C \leq 25\%$). Diversity

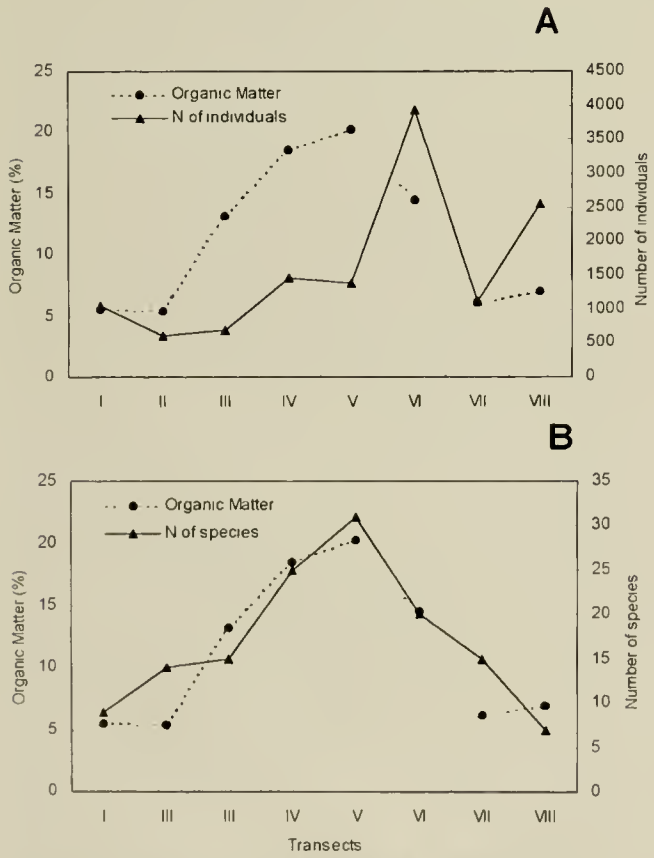

Figure 2. Total number of individuals (A) and species (B) as a function of organic matter in the sediment (percentage of dry weight) in each transect sample in Ubatuba Bay for whole year.

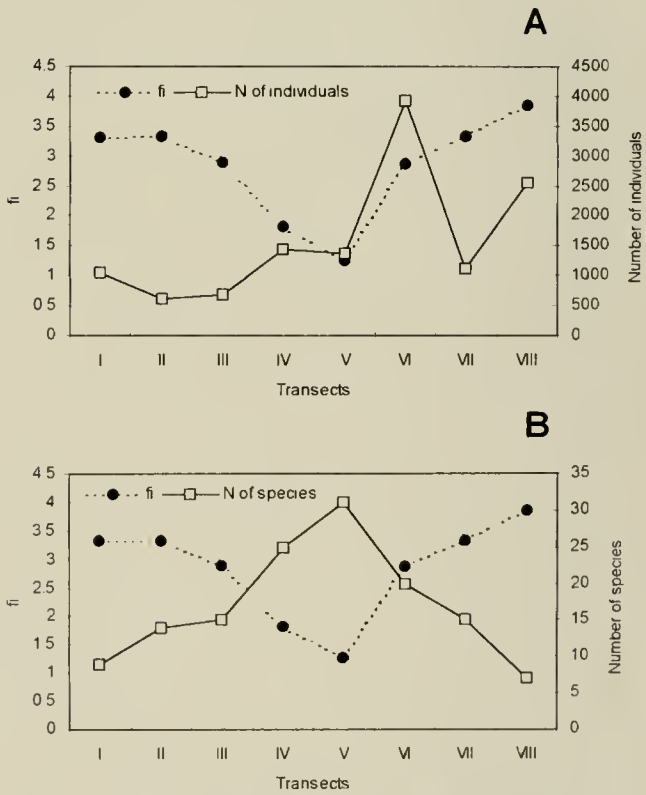

Figure 3. Number of individuals (A) and species (B) as a function of the central sediment tendency (fi = ϕ) in each transect sample of Ubatuba Bay for whole year. The ϕ was calculated according to Suguio (1973) from the formula $\phi = -\log_2 d$, where d = grain diameter (mm). The distribution curve was obtained by the formula ϕ 16 + ϕ 50 + ϕ 84/3.

was calculated using the Shannon-Weaver index (Shannon and Weaver 1963): $H' = \sum_{i=1}^{s} Pi. \log_2 P_i$, where "$s$" is the number of species and "P_i" is the proportion of i^{th} species. The equitability index (J') was calculated as indicated by García Raso and Fernandez Muñoz (1987): $J' = H'/\log_2 s$. Pearson coefficient was used to check relationships between the absolute values of each environmental factor studied and the number and frequency of species for all subareas combined.

RESULTS

A total of 12,790 brachyurans belonging to 50 species and 10 families was collected (Tables 1 and 2). The brachyuran taxocoenosis was dominated by *Callinectes ornatus* (60.4%), *Callinectes danae* (18.8%), and *Hepatus pudibundus* (7.7%), together representing 86.9% of the total collection. These three dominant species are differentially distributed in Ubatuba Bay (Table 1).

The greatest number of species was recorded for transects VI (30.8%) and VIII (20.0%). Species richness was significantly correlated with coarse grains sediments ($P = 0.00017$; $r = -0.96$) and with high organic content (Figs. 2 and 3). No correlation was observed between the above factors and number of individuals ($P = 0.00265$; $r = 0.90$). Pearson's analysis revealed significant coefficients between some species and group correlation (Table 3).

Continuous and heterogeneous occurrence throughout the sampling period was recorded for *H. pudibundus*, *P. punctata*, *A. cribrarius*, *C. danae*, *C. onatus*, and *P. spinimanus*. The number of species and their respective frequencies did not show significant seasonal variation, although both parameters increased slightly during the warmest months (Fig. 4) when the abundance of omnipresent species was greatest. In terms of their temporal pattern of occurrence, 26% of species were classified as omnipresent or constant, 18% as accessory, and 56% as accidental. The data of the most abundant species from monthly sampling taken on eight different subareas are shown in Table 4.

The diversity index ranged from 1.10 to 2.06 within transects and from 1.34 to 2.22 within months, depending more on equitability than on richness (Table 2 and Fig. 5). The lowest richness values were detected in transect VIII (7.0) which was influenced by fresh water inflow and significantly contrasted with those observed in transects IV (31.0), which had a high percentage of medium sand and organic content. The highest diversity and equitability were recorded in transects V (high percentage of medium sand and organic content) and I (highest depth and high percentage of fine sand), while the lowest values were obtained in transect VIII. During the study period both indexes showed wide variation along.

DISCUSSION

Examination of the species collected in Ubatuba Bay provided new information on the Brachyura fauna of the area and confirmed the biological potential of this region. Of all the brachyuran species recorded from Brazilian waters, 15.9% were found in Ubatuba

TABLE 3.

Coefficients of Pearson's Linear Correlation carried out between the abundance of total individuals (TO), the most abundant species, and the sampled environmental factors for all subareas combined.

	Coefficients											
Variables	**TO**	**Hp**	**Pm**	**Pp**	**Lf**	**Ac**	**Cd**	**Co**	**Ch**	**Ps**	**Hx**	**Hs**
Depth	0.135	−0.200	−0.174	0.171	−0.189	−0.173	0.094	0.178	0.225	−0.417	0.350	0.295
Dissolved oxygen	0.047	0.097	0.218	0.304	0.114	−0.398	−0.037	0.009	0.130	0.071	−0.234	0.412
Temperature	0.417	−0.436	−0.452	−0.678**	−0.670**	0.190	0.664*	0.339	−0.008	−0.174	0.363	0.607*
Salinity	0.123	−0.216	−0.056	−0.065	−0.147	0.106	0.252	0.010	0.360	−0.421	0.568*	0.362
Organic matter	−0.282	0.364	0.173	−0.008	0.056	−0.091	−0.391	−0.200	−0.327	0.230	−0.320	−0.297
Sediment												
Gravel	−0.275	0.087	0.005	−0.244	0.055	0.231	−0.118	−0.375	0.038	0.122	−0.086	−0.264
Very coarse sand	0.048	0.243	0.083	−0.088*	−0.065	0.503	0.106	−0.024	−0.360	0.096	−0.301	−0.313
Coarse sand	0.156	0.622*	0.501	0.160	0.133	0.205	−0.053	0.160	−0.567**	0.555	−0.628	−0.205
Medium sand	0.279	0.280	0.054	0.564*	0.422	−0.161	−0.019	0.387	−0.140	0.077	−0.159	−0.181
Fine sand	0.250	−0.477	−0.574	−0.268	−0.294	−0.112	0.269	0.359	0.253	−0.151	0.536	0.191
Very fine sand	−0.401	−0.371	−0.096	−0.404	−0.219	−0.378	−0.251	−0.321	0.028	−0.240	0.184	0.236
Silt and clay	0.141	−0.078	−0.023	0.453	0.228	0.005	0.159	−0.007	0.622*	−0.200	0.291	0.131
Species												
TO		0.311	0.164	0.246	0.054	0.524	0.749*	0.927*	0.031	−0.035	0.084	0.390
HP			0.879*	0.723*	0.669*	0.178	−0.265	0.414	−0.346	0.412	−0.481	−0.338
Pm				0.581*	0.663*	0.164	−0.288	0.197	−0.234	0.337	−0.436	−0.168
Pp					0.692*	−0.139	−0.316	0.357	0.105	0.377	−0.183	−0.336
Lf						0.115	−0.305	0.068	0.069	0.030	−0.262	−0.390
Ac							0.560*	0.388	−0.165	−0.289	−0.231	−0.089
Cd								0.504	0.095	−0.423	0.135	0.667*
Co									−0.079	0.110	0.105	0.168
Ch										0.087	0.705*	0.204
Ps											−0.077	−0.314
Hx												0.277

The pairs of variables show a tendency to increase (*) or to decrease (**) correlation together ($P < 0.05$). Hp, *Hepatus pudibundus;* Pm, *Persephona mediterranea;* Pp, *Persephona punctata;* Lf, *Libinia ferreirae;* Ac, *Arenaeus cribrarius;* Cd, *Callinectes danae;* Co, *Callinectes ornatus;* Ch, *Charybdis hellerii,* Ps, *Portunus spinimanus;* Hx, *Hexapanopeus paulensis;* and Hs, *Hexapanopeus schimitti.*

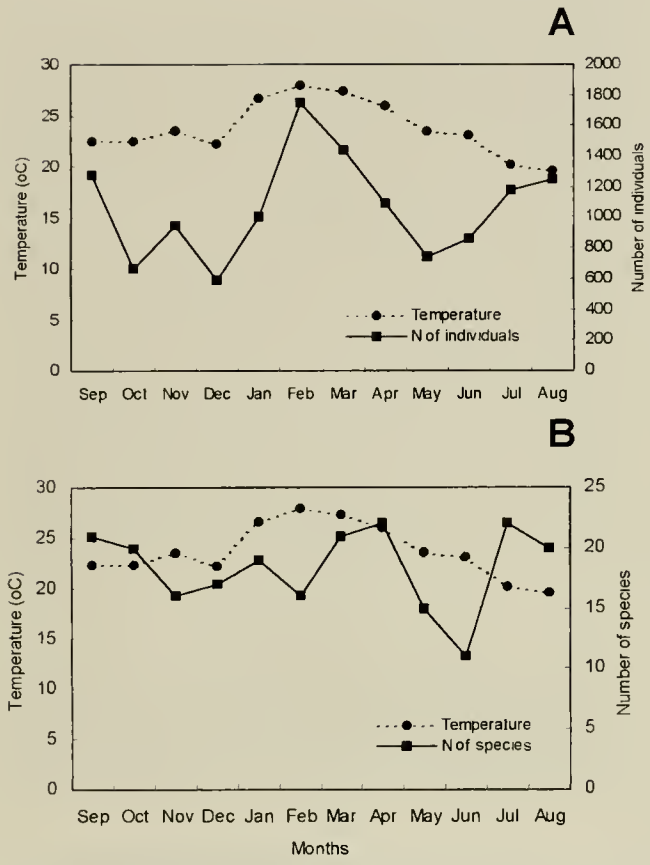

Figure. 4. Number of individuals (A) and species (B) as a function of temperature throughout the study period (September 1995 to August 1996) in Ubatuba Bay for transects and subareas combined.

Bay. As discussed by Fransozo et al. (1998) in a study of anomurans, the above percentage may be regarded as relatively high, considering the small area of this bay compared to the extent of the Brazilian coast. The present study revealed a diversity at least two times higher than that obtained in similar studies in the Ubatuba region carried out by Fransozo et al. (1992) in Fortaleza Bay, and by Negreiros-Fransozo and Nakagaki (1998) in Ubatuba Bay. It is likely that this difference was partly due to the sampling methodology (higher capture effort associated with the higher number of sampled subareas).

In all three study areas the dominant families in terms of numbers of individuals were the Portunidae and Calappidae. The largest temporal variation in species composition and density was strongly influenced by two species, *C. ornatus* and *C. danae*. The relatively large abundance of both species is probably due to their high fecundity as they have more than one reproductive cycle a year in this bay (Costa and Negreiros-Fransozo 1998, Mantelatto and Fransozo 1999b). Even though *C. danae* was common in the three aforementioned transects it only dominated the transect VIII collections. *Callinectes ornatus* was the most common brachyuran species in every other transect in addition to being common in transect VIII. This pattern was also found by Negreiros-Fransozo and Fransozo (1995) in Fortaleza Bay, adjacent to Ubatuba. *Callinectes danae* was most frequent in subareas 6, 7, and 8 influenced by freshwater. Euryhaline species such as *C. ornatus* and *C. danae* are found at both low and high salinity environments, as function of their growth, development, spawn, and larvae dispersion phases.

Among the environmental factors that influence the occurrence of brachyuran crabs in Ubatuba Bay, the sediment texture and organic content may be the most important agents. Both parameters accounted for the spatial distribution of *H. pudibundus* and *C. ornatus* in the Ubatuba region, studied by Mantelatto et al. (1995) and Mantelatto (2000), respectively.

Although environmental factors can delimit the distribution of benthic species (Pinheiro et al. 1996), their relative importance may differ among species, for the same species in different regions, or in the same region in different years. According to Fransozo et al. (1998), organic matter was deposited among sediment particles or laid over the substratum as a covering layer in Ubatuba Bay, with biogenic fragments mainly consisting of remains of polychaetes, mollusks, crustaceans, and echinoderms. In this study the organic matter content of the substrate was much higher than data reported previously for three other areas of Ubatuba region (see Mantelatto and Fransozo 1999a). Since organic matter has been known to play an essential role in benthic crustacean distribution, it along with abiotic conditions such as salinity, sediment size, and temperature, may determine the development and establishment of benthic invertebrates in Ubatuba.

One purpose of this study was to delimit both spatially and chronologically the brachyuran distribution in Ubatuba Bay so as to identify important parameters for experimental investigations to determine their relative influence on specific brachyuran species.

The number of individuals collected and the species diversity

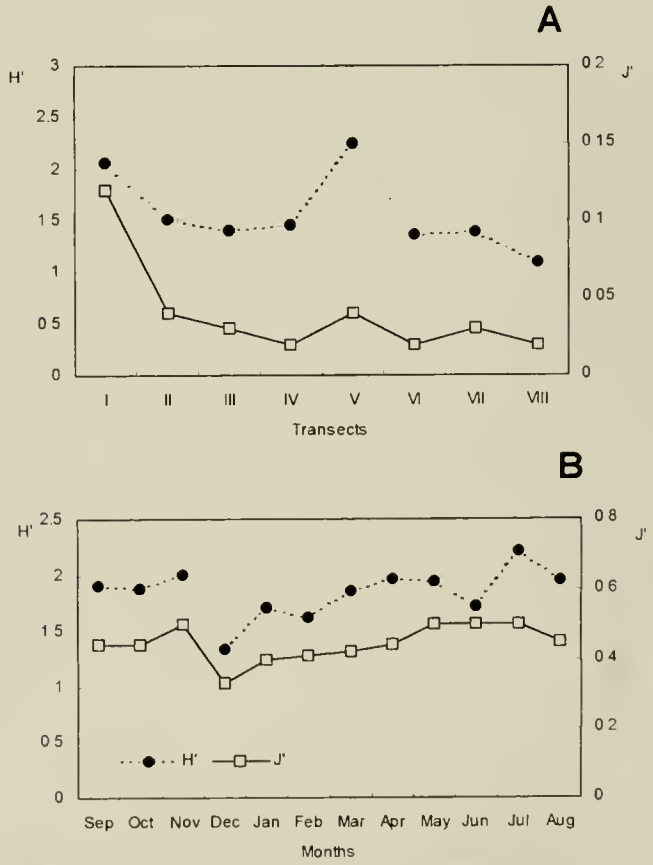

Figure. 5. Spatial and monthly oscillation of diversity (H') and equitability (J') for both each transect area (A) and month (B) during the study period (September 1995 to August 1996) in Ubatuba Bay for transects and subareas combined.

TABLE 4.

Number of individuals of the most abundant species from monthly sampling on eight subareas.

Subareas	Months												Total
	S/95	O	N	D	J/96	F	M	A	M	J	J	A	
Hepatus pudibundus													
I	32	20	17	6	16	60	30	–	1	18	55	11	266
II	9	4	5	2	11	4	5	2	1	2	–	5	50
III	20	5	8	4	21	1	1	1	1	7	12	5	86
IV	2	5	1	–	–	–	–	13	14	2	33	26	96
V	22	–	4	1	–	–	–	5	2	–	–	1	35
VI	45	47	71	18	20	16	17	18	8	20	25	63	368
VII	3	18	7	9	19	3	1	–	2	2	2	4	70
VIII	–	2	4	–	1	–	1	–	–	2	3	2	15
Persephona mediterranea													
I	19	8	17	3	14	3	8	–	–	1	24	8	105
II	–	–	–	–	–	–	–	–	–	–	2	–	02
III	–	–	–	–	–	–	–	–	–	–	1	–	01
IV	–	3	1	–	–	–	–	–	1	1	2	4	12
V	2	–	–	–	–	–	–	–	–	–	–	–	02
VI	–	–	–	–	–	–	1	–	–	1	–	1	03
VII	–	–	–	–	1	–	–	–	–	–	–	–	01
VIII	–	–	–	–	–	–	–	–	–	–	–	–	–
Persephona punctata													
I	1	–	4	–	–	2	1	–	–	–	3	9	30
II	1	–	–	–	3	–	–	–	–	1	–	–	05
III	2	–	–	2	–	–	–	–	–	–	–	1	05
IV	1	2	–	–	–	1	–	5	4	–	25	32	70
V	52	–	12	1	1	–	–	1	1	–	–	–	68
VI	13	15	27	2	2	8	4	6	4	6	25	69	181
VII	–	–	–	1	3	–	1	–	1	1	1	3	11
VIII	–	–	–	–	–	–	–	–	–	–	–	–	–
Libinia ferreirae													
I	–	2	1	–	–	1	–	–	–	–	1	–	05
II	–	–	1	–	–	–	–	–	1	–	1	4	07
III	–	–	2	1	–	–	–	–	–	1	–	1	05
IV	–	–	3	1	–	–	–	–	–	–	4	4	12
V	2	2	–	–	–	–	–	–	–	–	–	1	05
VI	–	–	4	–	–	–	–	2	–	–	3	–	09
VII	–	–	–	–	–	1	–	–	–	–	5	1	07
VIII	–	–	–	–	–	–	–	–	–	–	–	–	–
Arenaeus cribrarius													
I	16	17	19	8	8	49	28	1	12	23	22	10	213
II	15	8	5	2	11	14	7	3	13	9	7	4	98
III	–	–	1	–	1	–	–	–	–	–	–	–	02
IV	–	–	–	–	–	–	–	–	1	–	–	–	01
V	–	–	–	–	–	–	–	–	–	–	–	–	–
VI	–	–	–	–	–	–	–	–	–	–	–	–	–
VII	2	6	–	10	8	1	–	11	11	8	17	11	85
VIII	–	2	3	–	6	2	–	12	2	5	15	–	47
Callinectes danae													
I	1	–	–	–	–	2	–	–	–	–	1	–	04
II	1	1	–	1	1	1	4	1	1	5	–	–	16
III	5	2	3	–	–	5	23	4	15	16	11	2	86
IV	1	–	–	–	–	–	5	1	1	–	1	–	09
V	–	–	–	–	–	–	–	–	–	–	–	–	–
VI	20	20	38	7	5	34	33	35	30	72	56	43	393
VII	10	5	20	7	9	40	86	18	39	16	10	12	272
VIII	66	19	21	13	99	433	391	175	76	160	110	63	1626
Callinectes ornatus													
I	41	19	23	21	16	154	114	5	7	27	8	1	436
II	47	7	11	39	81	108	32	22	17	33	9	16	422
III	41	12	13	71	60	51	103	23	17	36	21	41	489

TABLE 4.

continued

Subareas	Months												Total
	S/95	O	N	D	J/96	F	M	A	M	J	J	A	
IV	42	52	47	58	5	2	5	277	155	43	164	261	1111
V	400	15	89	5	8	107	13	117	45	6	21	8	834
VI	215	236	359	173	296	230	246	157	134	198	309	357	2910
VII	25	48	28	72	126	110	65	12	38	38	42	53	657
VIII	13	37	22	27	104	269	114	49	17	85	74	55	866
Charybdis hellerii													
I	–	–	–	–	–	–	–	–	–	–	–	–	–
II	–	–	–	–	–	–	–	1	–	–	–	–	01
III	–	–	–	–	–	–	–	–	–	–	–	–	–
IV	–	–	–	–	–	–	1	1	–	1	1	–	04
V	07	01	06	–	–	–	15	36	32	1	14	19	131
VI	–	–	01	–	–	–	–	2	–	–	–	–	03
VII	–	–	–	–	–	–	–	–	–	–	–	–	–
VIII	–	–	–	–	–	–	–	–	–	–	–	–	–
Portunus spinimanus													
I	–	–	–	–	–	–	–	–	–	–	–	–	–
II	–	–	–	–	–	1	–	–	–	–	–	–	01
III	–	–	–	–	–	–	–	–	–	–	–	–	–
IV	14	–	5	6	8	–	–	9	16	3	2	2	65
V	47	14	29	–	5	3	2	14	5	8	6	10	143
VI	–	1	1	–	1	1	–	–	–	–	–	1	05
VII	–	–	–	–	1	–	–	–	–	–	–	–	01
VIII	–	–	–	–	–	–	–	–	–	–	–	–	–
Hexapanopeus paulensis													
I	–	–	–	–	–	–	–	–	–	–	–	–	–
II	–	–	–	–	2	–	–	1	–	1	–	–	04
III	–	–	–	–	–	–	–	–	–	–	1	–	01
IV	–	–	–	–	3	1	–	4	–	–	–	1	09
V	1	–	–	3	2	2	4	4	–	1	–	4	21
VI	–	–	–	–	–	–	–	9	5	–	1	–	15
VII	–	–	–	–	–	–	–	–	–	–	–	–	–
VIII	–	–	–	–	–	–	–	–	–	–	–	–	–
Hexapanopeus schmitti													
I	–	–	–	–	–	–	–	–	–	–	–	–	–
II	–	–	–	–	–	–	–	1	–	–	–	–	01
III	–	–	–	–	–	–	–	–	–	–	–	–	–
IV	1	–	–	2	–	4	5	12	–	–	–	–	24
V	–	–	–	1	9	1	23	1	–	–	–	–	35
VI	–	–	–	–	–	–	19	–	–	–	–	–	19
VII	–	–	–	–	–	–	–	–	–	–	–	–	–
VIII	–	–	–	–	–	–	–	–	–	–	–	–	–

increased during the summer months in the Ubatuba region. This can be explained by the interaction of two water masses, i.e. Coastal Water (CW) and South Atlantic Central Water (SACW), with temporal and spatial effects dependent on the penetration intensity of SACW. The SACW is rich in nutrients and when it reaches shallow areas in summer primary production increases causing pronounced eutrophication. Consequently, more food should be channeled to the benthos in summer, which would explain the seasonal variation in its biomass (Pires 1992).

We infer that both brachyuran community composition and diversity are controlled, at least in part, by seasonal abundance of dominant species associated with monthly changes in environmental conditions (Table 3). In this respect, intra- or interspecific factors (segregation of the sexes, competition, prey-predator relations, reproductive, and molt cycles, among others) could act to partition the resources of living space during a specific period when more food is available to adults or larvae (Mantelatto 2000). Alternatively, the presence of rare species such as *Collodes inermis*, *Podochela riisei*, and *Portunus ventralis* reported by Góes et al. (1998) might be the result of accidental introduction by offshore fishermen who sort their catch in Ubatuba Bay before taking it to commercial wholesalers.

Because of the large number of species and individuals documented in this study, it is difficult to explain in full the brachyuran distribution in this bay, but we may infer, as reported for *Callinectes ornatus* in a previous study, that the presence or absence in an area results from interdependence between phases of ontogenetic development and the conditions of the physical environment (Mantelatto 2000). Field observations on the extent of wave intensity, buoy movements, fishing sites, commercial trawling activity, scuba diving, and deposition of particles in the subareas implied the existence of a strong circulation, with a predominant

inflow reaching successively the following subareas: 1→2→7→8. Mantelatto and Fransozo (1999a), found subareas 4 and 5 to be reproductive sites judging by the numerous ovigerous females of Brachyura and Anomura collected there, suggesting that these subareas are favorable to brooding and larval dispersion. The greater number of species (31) in subareas 4 and 5 (southern portion of bay) and the presence of 22 species found nowhere else in the bay probably is related to the higher density of biogenic fragments and the proximity of a steep, protective coastal shoreline. This idea is supported by the presence of predominant species such as *A. violaceus*, *N. brasiliensis*, *S. seticornis*, *C. hellerii*, and *P. reticulatus*, which live in consolidated habitats (reefs) or in association with algae, and not in the non-consolidated area. In this way the abiotic conditions and intra- or interspecific relationships may be cross-correlated and lead to the distribution observed.

The present study documents the distribution of some 50 species of Brachyura found in Ubatuba Bay and points out the need for more detailed studies on the environmental parameters, biodiversity, larval dispersion, and larval settlement in different biotopes to improve knowledge of the underlying factors determining population structure and dynamics of the brachyuran community of this important faunal transition zone on the northern coast of São Paulo State.

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LABORATORY OBSERVATIONS ON THE REPRODUCTIVE AND MOLT CYCLES OF THE ROBINSON CRUSOE ISLAND LOBSTER *JASUS FRONTALIS* (MILNE-EDWARDS, 1836)

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ABSTRACT Observations on molting, mating, embryo- carrying, hatching, and early larval development were made on the Juan Fernández lobster *Jasus frontalis* maintained in seawater aquaria at the Coastal Aquaculture Center of the Universidad Católica del Norte, Coquimbo, Chile. Males underwent their annual prereproduction molt from February through March at temperatures around 17 °C and females molted from mid-April to mid-June at temperatures of 13 to 15 °C. Growth at each molt was about 1.3 to 3.1 mm in both males and females, and hardening of the carapace occurred over 3 days. Embryo- carrying was initiated between the months of June and August, with hatching of larvae between October and December. Embryonic development lasted 115 days at 13 °C and 76 days at 18 °C. Hatching released a naupliosome larvae normally occurring between sunset and before midnight. The naupliosoma swam actively for 10–20 min, then molted to produce the first phyllosome larvae. Five days after hatching, 2–8 % of the egg mass remained attached to the pleopods of the females, as did numerous empty capsules. These remaining eggs were in the first stage of development. Six stages of larval development were obtained. The six larval stage were observed after 56 to 92 days, depending upon incubation temperature. At 14–15 °C the fifth stage was obtained at 77 days; at 17–18 °C the sixth stage was obtained at 79 days, and after 56 days at 20–21 °C.

KEY WORDS: *Jasus frontalis* lobster, reproductive cycle, molt

INTRODUCTION

Jasus frontalis,the spiny lobster endemic to the Juan Fernandez Archipelago, is a commercially valuable decapod crustacean in Chile's oceanic waters. It has been exploited since about 1893, thus constituting one of Chile's oldest fisheries. However, the annual catch of this resource has decreased from 140 tons in 1964 to 20 tons in 1996 (Arana and Toro 1985, SERNAP 1996) despite management measures implemented in the 1960s (Arana and Toro 1985, Yáñez et al. 1985). This decline emphasized the need for new research on mating, spawning, and larval development that would permit the design of more adequate regulations for the protection of this species.

Early research on this lobster was oriented primarily toward such fisheries-related parameters as growth (Arana and Martínez 1985), mortality and yield (Diaz and Arana 1985), capture (Arana and Melo 1973, Larraín and Yáñez 1985), and population structure and dynamics (Gaete and Arana 1985, Yáñez et al. 1985). Little is known about its reproduction and development. Arana et al. (1985) determined size at first sexual maturity and fecundity, and described 10 stages of embryonic development on the basis of morphological characteristics and degree of coloration of the embryos. The description of some of the larval stages (VII, XI, XIII A and B) was made from plankton samples obtained near the Robinson Crusoe island (Baez 1973).

More recent research has begun to elucidate the reproductive biology of this species. Each of the stages of embryonic development has now been described using light (Dupré 1988) and scanning electron microscopy (SEM) (Tavonatti 1998). The structural organization of the ovary was described by Elorza (1998). The duration and characteristics of different phases of the molt cycle as revealed by analysis of pleopods, were described by Elorza and Dupré (1996).

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Information on larval biology of the species is scarce and recent. Dupré (1996) described the first stage phyllosoma larva and later observed the first five phyllosoma stages in experimental cultures over a 3-month period (Dupré and Guisado 1996). Larval cultures were subject to high mortalities because of infection of larvae by *Vibrio* spp. and filamentous fungi (Dupré unpublished data).

The present study evaluates the key events in the complete reproductive cycle of this species, establishing the chronological sequence of these events using specimens in captivity. The molting period in males and females, mating period, time between mating and appearance of embryo masses (berrying), periods of hatching of larvae, and also the periods of embryonic and larval development at different temperatures were observed. Our laboratory observations were made with the intention of duplicating the reproductive cycle as it occurs in nature.

MATERIALS AND METHODS

Lobsters were obtained from Robinson Crusoe island (33 ° 40′ S; 78 ° 40′ W) between 100 and 150 m depth and transported to the Coastal Aquaculture Center of the Universidad Católica del Norte at Coquimbo (29 ° 58′ S; 71 ° 22′ W).

Females and males over the first sexual maturity size (cephalothoracic length, CL = 75 mm for females and 82 mm for males; Arana et al. 1985) were studied. Specimens included 31 females (CL = 85.5 to 99.2 mm) and 16 males (CL = 92.6 to 116.7 mm). Specimens were obtained in 1994 and 1995, and experimentation was carried out in 1995 and 1996. Almost no mortality occurred during the experimental period.

Males (M) and females (F) were distributed into four circular 500-L seawater tanks with constant flow of 50 μm filtered seawater at ambient temperature, which ranged from a minimum of 13.1 °C (6/95) to a maximum of 17.8 °C (12/96). Continuous aeration was provided to each tank. The distribution of the specimens was as follows: Tank 1–7 F, 7 M; Tank 2–9 F, 5 M; Tank 3–9 F, 3 M; and Tank 4–6 F and 1 M with a cephalotoracic lenght

(LC) of 116.7 mm. This male was able to fertilize three females previous to the experiments.

The tanks were inside a laboratory greenhouse with ambient light regime. The tanks were covered with shade netting that reduced total light entry by about 50 %. The lobsters were fed with frozen fish *ad libitum* every 2 days for 2 years. Unconsumed residues and feces were removed from the tanks on each day following feeding.

Tanks 1–3 were observed daily for evidence of molting; molts were removed, and molted individuals were measured and transferred to individual 50-L tanks until their carapaces had hardened. Later, each one of the molted female (n = 8) were transferred to Tank 4, where they were observed every 4 hours each day to detect mating or presence of eggs on pleopods. The size increment of recently molted individuals was measured, always using the length from the postorbital margin to the dorsal termination of the cephalothorax.

Embryonic development was determined at four different temperature ranges: 12–14 °C; 15–17 °C, and 17–19 °C. Each embryonic development stage was determined according to Dupré (1988), by light microscopy, of 10–15 embryos obtained from the medial region of the abdomen of six ovigerous females carrying 225,350 to 287,200 embryos

To determine larval hatching time, females with embryos in final developmental stages (Dupré 1988) were placed in 50-L tanks to capture larvae upon their release. Seawater flowing out of these tanks was passed through semisubmerged 300-μm screens on which recently hatched larvae were captured. Larvae recovered from these screens were washed in 10-μm filtered seawater and placed in 1-L glass beakers. Temperatures in the hatching observation varied from 15 ± 1 °C to 19 ± 1 °C.

Larval cultures were also carried out at four different temperature ranges: 14–15 °C, 17–18 °C, 19–20 °C, and 20–21°C maintained by a Jager thermostat. The water was changed daily in each beaker, and larvae were fed *ad libitum* with *Artemia nauplii*. Larvae were observed daily in the microscope to follow developmental stages. Dead larvae lost the transparency and were observed opaque-white. They were removed by a glass tube. To determine the development time of the different stages, 325 larvae distributed in eight 1-L glass beakers in groups of 30–50 larvae per L, were used.

RESULTS

Molting and Reproductive Cycle

The annual reproductive and molting cycle is diagrammed in Figure 1. Molting in males occurred between February and March each year, when the temperature reached an average of 17 ± 0.5 °C (1995) and 16.9 ± 0.4 ° C (1996). Females molted from the middle of April to the middle of June, when temperatures reached an average of 15.0 ± 0.5 °C (1995) and 13.1 ± 0.2 °C (1996). Growth (in CL) per molt varied between 1.4 and 3.6 mm in males and 2.2 and 3.1 mm in females; average increase by males was 2.3 and females 2.7 %. Hardening of the carapace occurred slowly over 3 –5 days, during which the individual ceased feeding and showed little activity.

Mating

Because no mating was observed during the day, it was concluded that copulation occurred at night. In most cases, mating occurred between postmolt females and males of a larger size. When males with CL = 92.6 and 102.6 mm were presented to CL = 95 and 99.2 mm, respectively, recently molted females, none were observed to be carrying embryos on their pleopods. In two cases, using a CL < 95 mm males, eggs were found on the bottoms of the aquaria, with minor amounts of eggs (100–300) adhered to the pleopods of the females. The male in Tank 4 (CL = 116.7 mm) was the only one able to fertilize the oocytes of the postmolted females; it fertilized all six molted females introduced into the tank between June and August. All females presented viable embryos on the pleopods; these developed normally.Although no mating was observed, this was estimated to occur sometime between 3 and 20 days postmolt, because egg-bearing females were observed at 21 days postmolt.

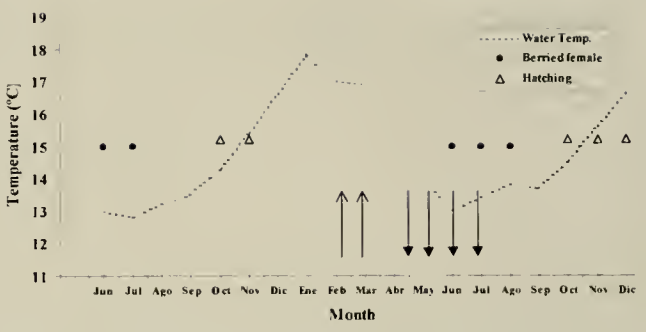

Figure 1. Reproductive and molting cycle of the spiny lobster of Juan Fernández *Jasus frontalis*, reared in the laboratory. Key : male molt (↑), female molt (↓), embryos carrying females (●), batching of larvae (△), temperature of the tank water (Temp.).

Spawning

Recently spawned eggs were spherical, with diameters of 590–610 μm. They were covered with an adhesive coating (chorion or vitelline coat), separate from the plasma membrane (Fig. 2a), which allowed them to adhere to the pleopods and later formed the funiculus. At the beginning of adhesion, the funiculus was short (120– 250 μm) and wide (450–500 μm) (Figs. 2b, c); within 30 to 40 min, eggs were observed adhering to the setae of the pleopods (Fig. 2d) with the funiculus thinner (30–40 μm) and three to four times the initial length.

Embryonic Development

The total embryonic development, from spawning to the VII stage, occurred between 115 and 76 days at temperatures average of 13 ° C (ranging 12–14 °C) and 18 °C (ranging 17–19 °C), respectively (Fig. 3). Average times of development were 76 (range ± 6) days at 18 °C (range 17–19 °C); 85 ± 4 days at 16 ± 1 °C and 115 ± 3 days at 13 ± 1 °C. The major variation in development time with temperature was observed with the first stage (29 days at 13 °C, 22 days at 16 °C, and 19 at 18 °C). Between developmental stages IV and VII, variations caused by temperature were not significant when incubated at 16 °C (average = 10.5 days; SD = 1.4) and 18 °C (average = 10.0 days; SD = 1), but was significant between stages II and IV at 13 °C (average = 12 days; SD = 0.2) and 18 °C (average = 8.0; SD = 0.4).

Hatching

The first larval stage to hatch from the egg is the naupliosome (Dupré 1996). After 20 min of active swimming using the ex-

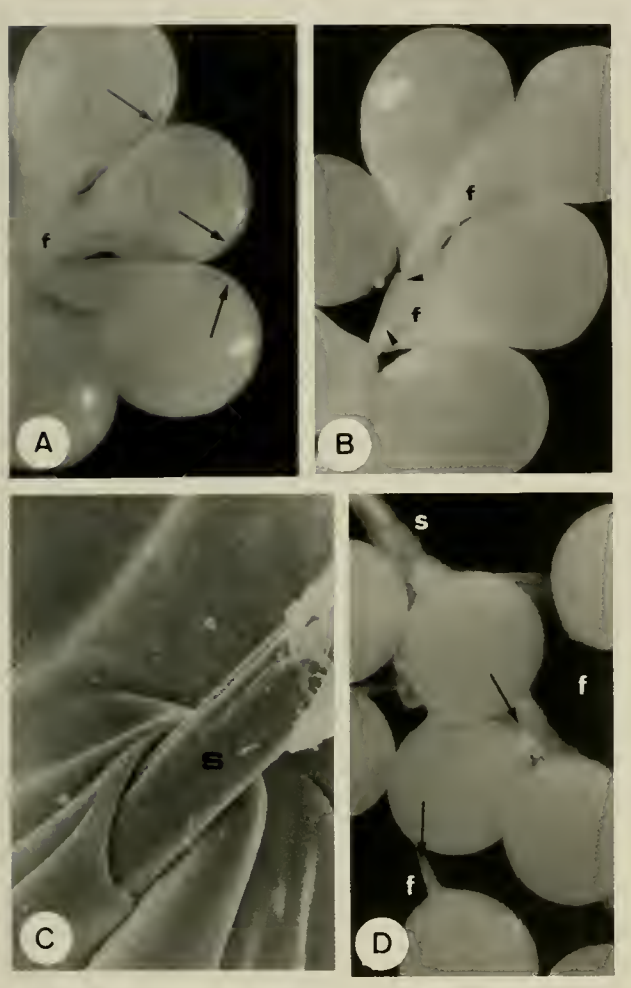

Figure 2. Recently spawned eggs adhered to pleopods setae. (A) Shows the highly adhesive chorion at the beginning of the adhesion; initially it is separated from the surface of the egg (arrows). The outer side of the chorion has adhered to the setae of the pleopod and begun to stretch (54X) . (B) Eggs adhered to the setae of the pleopod 5 min postspawn. Note chorion attached to the seta (arrowhead) without formation of a funiculus (54X). (C) Scanning electron microscopy (SEM) view of the chorion adhered to a setae (s) (1,100X). (D) Eggs adhered to a seta (s) of a pleopod forming a funiculus (f and arrows) 30 min after spawning (50X).

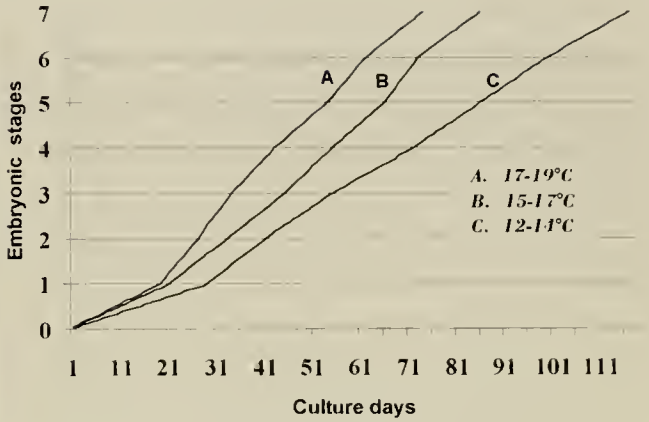

Figure 3. Embryonic development at different temperatures.

opodites of the antennulae, these molted to produce the first phyllosome larvae. Hatching of larvae occurred between October and November in each year of study, and in all cases occurred after sunset and before midnight. Female lobsters actively initiated the hatching process by extending and raising the abdomen to about 20° above horizontal and rhythmically agitating the pleopods to produce a water current, which expelled larvae posteriorly.

The larvae were strongly phototactic, swimming toward the surface, or toward any light source entering their environment. Pleopodal beating of the females was active three to five times at intervals of 30 min during the night. Activity suspended during daylight hours. This induction of hatching was carried out over 3 to 5 consecutive days, with most larvae expelled during the first 3 days. Although natural hatching normally occurred during the early evening, naupliosomes could be collected at any time by removing the female lobster from the aquarium and directing a gentle stream of seawater over the pleopods for 5 to 10 sec into a suitable receptacle (bucket). This method could be employed two or three times a day without adversely affecting the females. At the beginning of the hatching, embryos at different stages of development were observed (Fig. 4a). Five days after initiation of hatching, about 2–8 % of the egg mass was still adherent to the pleopods together to all the empty capsules left by enclosed larvae. The notable of those remaining eggs had not progressed beyond the first developmental stage (Fig. 4b) and they looked normal.

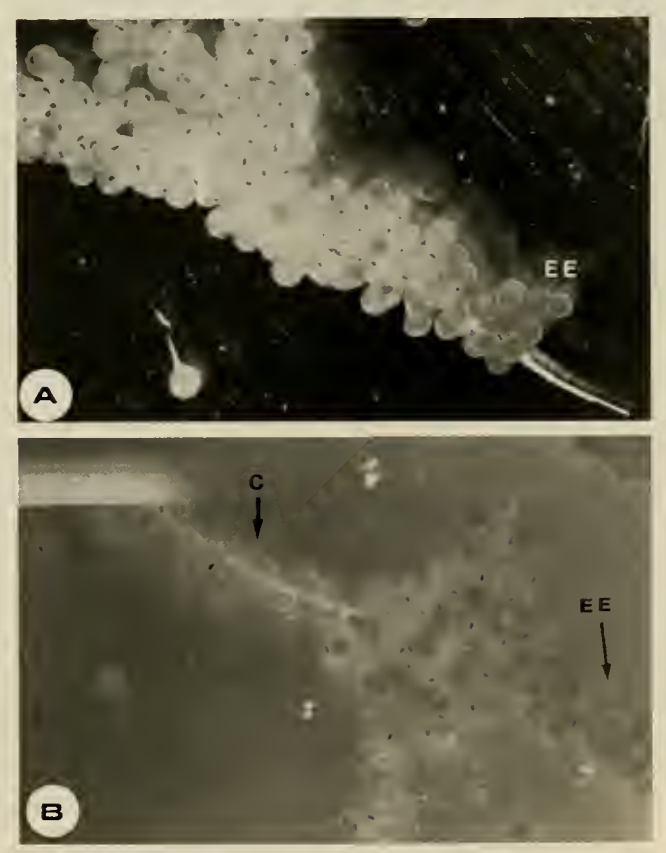

Figure 4. Embryos adhered to setae of a pleopod at the beginning of hatching. (A) Shows early developed embryos (EE) and final developed embryos at the distal end of the setae (8.3X). (B) Embryos adhered to a seta of a pleopod 2 days after the initial hatching. Observe empty capsules (C), early developed embryos (EE), and final developed embryos adhered to the same setae.

Larval Development

Six different stages of larval development were observed between 56 to 92 days in culture, depending upon temperature (Fig. 5). Cultures carried out at 20–21°C and 14–15 °C presented significant differences between the time required to reached stage VI and the time required in each development stage (average = 9.5 days per stage; SD = 1.04 and 15.3 days per stage; SD = 12.2, respectively). However, at 17–18 °C and 19–20 °C the difference between the averages time on each development stage was not significant (13.0 days, SD = 4.1 and 11.5 days, SD = 4.4, respectively). At all temperature ranges, the first two stages usually required longer time periods for development than the intermediate stages (III to IV), but less time than stages V and VI. Duration of the first phyllosome stage was similar, between 17 and 20 °C (12 to 19 days). In contrast, at 14–15 °C their duration was 38 days. After the second developmental stage, duration of each stage was directly related to culture temperature (Fig. 5).

Filtration of culture water to 10 μm was insufficient to prevent infestation of larval cultures with filamentous fungi, which adhered to larval pereiopods, antennae, antennulae, and buccal structures, and especially to the setae on swimmerets exopods of the pereiopods. The best larval survival and most regular periods between development stages (9.5 days in average) were obtained at 20–21 °C (Fig. 5)

DISCUSSION

Molting

Molting of male lobsters occurring in the summer months of February to March coincides with the beginning of seasonal temperature decline typical of their natural habitat (17.8–16.8 °C) (Neshiba and Silva 1985). Females began molting at the end of April and beginning of May, typical of when habitat temperature had declined to about 14 °C and onward through June as the temperature droped to about 12.9 °C, after which habitat temperature began to rise in July.

Arana and Martínez (1985), based on commercial captures in the Robinson Crusoe Island, reported the highest percentages of soft (recently molted) lobsters of both sexes were observed in January and February, and in September, with a notable presence of postmolt males in December to January. In our observations, molting commenced about one month later (Feb,–March).

This difference of molting periods between the captive specimens in aquaria and specimens in the field could be explained by the gradual softening of the branchiostegal plates that start from 35 days before molt (Elorza and Dupré 1996), which can produce errors in the estimation of the molt stage when it is determined by palpation of the branchiostergal plates of the females in the field; that is, field reports are based on different observations, and they include the premolt stage within the postmolt stage.Observations by Arana et al. (1985) that postmolt females are found in the environment in May and June do not coincide with our observations in the laboratory.

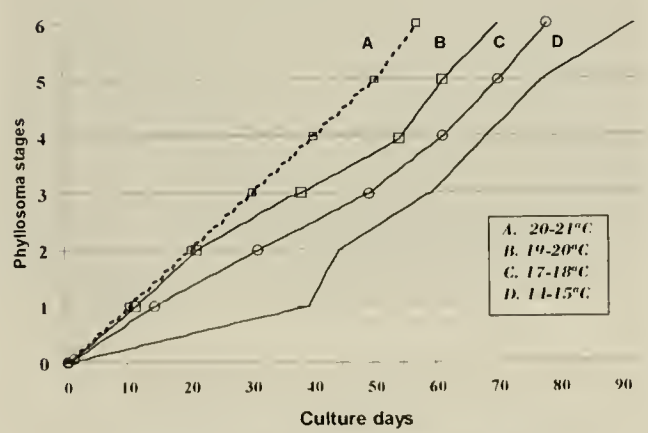

Figure 5. Early larval development at different temperatures.

Our values for cephalothoracic increases in length after molting (1.5–3.6 mm) were lower than those made indirectly through the analysis of the distribution of cephalothoracic length frequency, as estimated by Arana and Martínez (1985) for the same species (8.9 mm). Our results represent the first time growth data have been obtained by direct measurement. However, they estimated that length increasing per molt is constant to all the molt stages, which represents a decreasing rate per consecutive period of molt. Further support for this position was that the growth values estimated in our study were similar to those obtained for *Jasus lalandii* (3.0 mm for males and 1.0 mm for females with 6.0 to 9.9 cm cephalothorax) (Beyers 1979) and *Panulirus interruptus* (Mitchell et al. 1969), where the annual cephalothoracic length increment was 3.7 mm for male and 4.4 for females, using individuals from 5.1 to 9.1 cm of cephalothoracic length.

Following ecdysis, the carapace of *Jasus frontalis* has a soft texture, which begins to disappear on the first day postecdysis to the third to fifth day, when it has again become rigid. This result is considerably different from observations made on *Jasus lalandii* by Matthews (1962) and Heydorn (1969); the former author observed the soft-textured condition lasted for 14 days; whereas, the latter author estimated its duration at 15 to 32 days. The difference between the cited results and our present results, other than interspecific variability, may be attributed to the abundant diet (rich in Ca^{++}) given to lobsters in our laboratory or to an error in the determination of the molt stage by the compression of the branchiostegal plates of lobsters, as mentioned above.

Mating and Spawning

Although we were unable to witness copulation in relation to the precise time of molting, eggs appeared on the females about 21 days after males were placed with molted females. Mating occurred between individuals of similar size or with males larger than females. This observation may be of value in further attempts to culture this species, especially because we noted that the sexually mature male of over 95 mm cephalothoracic length (Methods: Tank 4) was able to fertilize at least six females in a period of 2 months.

Berried Phase

Our observations that females carried eggs from June through October coincided partially with observations of Arana et al. (1985) at Robinson Crusoe Island, who observed berried females from July to February of the following year with a period of maximum incidence between October and December. Our results showing precocious embryos-carrying females of laboratory specimens may be attributable to early molting, because maximum ovarian development is obtained in the period immediately following ecdysis and is promoted by the hormone vitellogenine, which is intimately related to the molting cycle (Meusy and Payen

1988). The advancement of molting, compared to that observed in the natural habitat (Arana et al. 1985), may be attributable to the abnormal rise in temperature experienced by specimens in the laboratory. Lobsters in their natural habitat (100–200 m) experience less variation in, and slower rates of change of temperature than those kept in laboratory tanks.

Hatching

Activity of the female lobsters, which produces liberation of larvae (agitation of pleopods) suggests presence of an adaptive mechanism to aid larvae unable to release themselves from the chorion that envelops them. Simple mechanical agitation of the pleopods presumably permits the releasing of the larvae.

Correct estimation of the fecundity of a species allows estimation of the number of larvae available for recruitment into the plankton. Previous estimates of this datum may be in error given that our observations show that 2–8 % of the eggs carried by females of *J. frontalis* may remain undeveloped on the pleopods (they remain in the first stage) after most larvae have been released. Thus, the real contribution to the larval population made by a female of 90–94 mm in cephalothoracic length that carries 131,000 to 301,000 eggs (Arana et al. 1985) would be diminished by 2,620–6,020 (2 %) to 10,480–24,080 (8 %) larvae, plus an unknown number of eggs lost during the normal incubation period and the normal estimation error of the fecundity. In *Palaemon pandaliformis* and *Macrobrachium acanthurus* the loss of eggs may reach 23 % (Anger and Moreira 1998).

As mentioned above, the presence of undeveloped eggs attached to the pleopods after 76–115 days of incubation presumably without deterioration of the yolk mass must be investigated. Because these oocytes were attached to the proximal end of the pleopods setae, it suggests they were not fertilized.

Larval Development

In the temperature ranges of our study, development of embryos and larvae was inversely related to culture temperature. Mortality was not the same at each temperature range observed, with larvae showing highest survival at 19–20 °C. This may be attributable to the higher frequency of molting in the higher temperature ranges. Molting avoids formation of filamentous fungi on setae of the exopodites of the pereiopods and the rest of the body, allowing more freedom of motion and better feeding efficiency.

We conclude from our results that the reproductive cycle of this lobster in captivity may not differ greatly from that observed in its natural habitat. Further research is recommended to complete the details missing for this valuable lobster species.

ACKNOWLEDGMENTS

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COMPENSATORY GROWTH RESPONSE FOLLOWING PERIODS OF STARVATION IN CHINESE SHRIMP, *PENAEUS CHINENSIS* OSBECK

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ABSTRACT The effects of previous periods of starvation on the subsequent changes in body mass, food consumption, food utilization, and body composition in Chinese shrimp, *Penaeus chinensis* were investigated. Upon refeeding, shrimp responded to various periods (4, 8, and 12 days) of food deprivation by exhibiting hyperphagia. There were the characteristic patterns that the intensity of compensatory appetite increased in proportion to the length of the starvation periods and that the hyperphagic responses of the starved shrimp were not sustained, and within 8 days declined to levels not significantly different from those of the controls fed continuously at satiation feeding. During subsequent refeeding, the previously 4-day-starved shrimp were only slightly higher, and the 8- and 12-day-starved shrimp were significantly higher in specific growth rates in terms of dry matter, protein, and energy content (SGR_d, SGR_p, and SGR_e) than those of the controls. However, there were no significant differences in SGR_w (SGR in terms of wet weight) among all the experimental groups. During the course of refeeding there were no significant differences in food conversion efficiencies (FCE_w, FCE_d, FCE_p, and FCE_e) among all groups, and only the shrimp previously starved for 8 days showed slightly higher FCEs (FCE_d, FCE_p, and FCE_e) than the controls. There was a trend that, within the first 8 days of refeeding, FCE_w decreased with the length of starvation periods, which may be attributable to changes in body water content. With food deprivation, lipid, protein, and energy content decreased and water content increased. At the end of starvation the shrimp starved for more than 8 days showed significantly lower lipid, protein, and energy content and higher water content than the controls. After 32 days of refeeding no significant differences in water, protein, and energy content were found between the starvation-satiation shrimp and the controls, except that lipid content of the shrimp starved for 4 or 12 days was still lower than that of the controls. The results of this study suggest that the shrimp regulate their appetite and growth rate in relation to their previous nutritional history.

KEY WORDS: *Penaeus chinensis*, compensatory growth, starvation, food utilization

INTRODUCTION

Aquatic animals, either in natural environments or under culture conditions, occasionally experience starvation or undernutrition. The capacity to withstand and recover from nutritional stress is an important adaptation for survival, growth, development, and reproduction of any organism that must sporadically endure periods of limited food supply. Thus to investigate the recovery growth in aquatic animals following a period of starvation or malnutrition is not only of theoretical value in ecophysiology and evolution (Russell and Wootton 1992, Nicieza and Metcalfe 1997), but also of important applications in aquaculture (Quinton and Blake 1990, Hayward et al. 1997). In crustaceans there have been a considerable number of reports concentrating on survival, development, and changes in metabolic activity and energy reserves during the period of starvation in attempts to characterize the physiological and biochemical effects of starvation (Anger et al. 1981, Barclay et al. 1983, Dall and Smith 1986, Dawirs 1987). By contrast, few studies have concerned on changes in biochemical composition (Whyte et al. 1986, Stuck et al. 1996), weight gain (Bostworth and Wolters 1995), and food consumption (Paul et al. 1994) during recovery growth following transfer to plentiful rations.

As noted by Cui (1989), many animals subjected to variable environment have the ability to actively regulate their growth. When refed following a period of undernutrition animals will often display a rapid growth spurt known as compensatory growth or catch-up growth. Compensatory growth is known to occur in a wide range of domestic mammals and birds (Wilson and Osbourn 1960, Mersmann et al. 1987, Yu et al. 1990). Amongst aquatic animals compensatory growth has mainly been reported for fish (Bilton and Robins 1973, Weatherley and Gill 1981, Dobson and Holmers 1984, Quinton and Blake 1990, Russell and Wootton 1992, Jobling et al. 1993, Jobling et al. 1994, Hayward et al. 1997). However, little information on compensatory growth in crustaceans is available (Bostworth and Wolters 1995). Moreover, there is no further evidence on physiological mechanisms of compensatory growth in crustaceans, although in general, compensatory growth in fish and other animals may be a result of hyperphagia, improving food conversion efficiency, or both on subsequent realimentation.

This study described the changes in weight gain, feed intake, food conversion efficiency, body composition, and energy content in Chinese shrimp, *Penaeus chinensis* Osbeck subjected to various periods of starvation following by satiation feeding with the aims to investigate the capacity for intrinsic growth regulation—compensatory growth response displayed by the shrimp and to determine the extent to which the relative severity of starvation influenced the subsequent growth and the restoration of body composition.

MATERIALS AND METHODS

Rearing Condition

Chinese shrimp, *P. chinensis* were kept in glass aquaria (45 × 30 × 30 cm, water volume of 35 L). Each rearing unit was stocked with 4 shrimp. The room temperature was controlled using an air conditioner. Aeration was provided continuously and one-half to two-thirds of volume water was exchanged every other day to

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ensure high water quality. Seawater used in the experiment was filtered by composite sand filters. During the course of the experiment, dissolved oxygen was maintained above 6.0 mg/L, the pH was around 7.8, water temperature was 25.0 ± 0.5 °C, the salinity of seawater was within 30‰ to 33‰, and a simulated natural photoperiod (14 h of light:10 h of darkness) was used.

Source and Acclimation of Shrimp

The experiment was carried out between August 26 and October 9, 1999 at the Laboratory of Aquacultural Ecology, Ocean University of Qingdao, Qingdao, People's Republic of China. The shrimp used in the experiment were collected from the Fengcheng Shrimp Farm, Qingdao. Prior to the experiment the shrimp were transferred into aquaria and underwent a 10-day acclimation period during which they were fed polychaete worms, *Neathes japonicus* (Izuka) at satiation level twice a day (at about 8:00 and 20:00).

Experimental Design

The experiment lasted for 44 days and was divided into two periods: the food deprivation period and the refeeding period. Four feeding groups were established: (1) Group C (control) was fed to satiation twice a day (at 8:00 and 20:00) for 32 days; (2) Group S4 was starved for 4 days followed by 32 days of satiation feeding; (3) Group S8 was starved for 8 days followed by 32 days of satiation feeding; and (4) Group S12 was starved for 12 days followed by 32 days of satiation feeding.

After 24 h of food deprivation, 114 shrimp with an initial wet body weight of 2.164 ± 0.055 g (mean ± SE) were selected of which 84 shrimp were randomly assigned to 21 aquaria using randomized complete block design with the four treatments (3 aquaria for the control group and 6 aquaria for each of the three starved groups). Each aquarium was stocked with 4 shrimp.

During the period of starvation, shrimp in Group S4, S8, and S12 were placed individually in sections of aquaria divided into quadrants by opaque plastic plates with holes (1 cm in diameter). The plates were removed at the end of starvation. During the refeeding period shrimp in all the four treatments were individually weighed every 8 days.

Samples Collection and Analysis

Three groups (10 shrimp each) were sampled from the originally selected batch for measurement of initial body composition. At the end of starvation, three aquaria of Group S4, S8, and S12, respectively, were randomly sampled and were weighed individually. Thus there were three aquaria that remained within each of the three groups previously subjected to starvation during the course of refeeding. After 32 days of refeeding the shrimp of all groups were starved for 24 h and then sampled. The shrimp from the same aquarium were pooled as a sample.

During the course of the experiment the daily food (polychaete worms) supplied was recorded and uneaten food was collected before the next ration was provided. To remove excess moisture, shrimp and food were carefully blotted with paper towel and weighed to the nearest 0.001 g using an electronic balance.

After the weight was obtained all the samples of shrimp and food were dried timely in an oven at 70 °C to constant weight, homogenized with a glass mortar, and stored at −20 °C. Before chemical composition analysis the samples were re-dried at 70 °C to constant weight.

Nitrogen content was measured using a PE-240C elemental analyzer and crude protein content was calculated from nitrogen content by multiplying nitrogen content by 6.25. Crude lipid was determined by the Soxhlet method (Osborne and Voogt 1978), ash was determined by combusting dried samples in a muffle furnace at 550 °C for 12 h, and gross energy content of dried samples was determined by bomb calorimeter (Cui et al. 1996). Analyses of each sample were conducted in triplicates.

Calculation of Data

Specific growth rate (SGR), feed intake (FI), and food conversion efficiency (FCE) in terms of wet weight were calculated as follows:

$$SGR_w\ (\%/day) = 100 \times (\ln W_2 - \ln W_1)/T$$

$$FI_w\ (\%\ body\ weight/day) = 100 \times C/[T \times (W_2 + W_1)/2]$$

$$FCE_w\ (\%) = 100 \times (W_2 - W_1)/C$$

Where W_2 and W_1 are the final and initial wet weight of the shrimp, T is the duration of growth period in days, and C is the weight of food consumed.

SGRs, FIs, and FCEs in terms of dry matter (SGR_d, FI_d, and FCE_d), protein (SGR_p, FI_p, and FCE_p), and energy content (SGR_e, FI_e, and FCE_e) were calculated similarly.

Energy content of protein and lipid in shrimp was calculated using factors 18.075 and 39.581 kJ/g, respectively (Schafer 1968).

Statistical Analysis

Statistics were performed using SYSTAT statistical software (SYSTAT 1992) with possible differences among groups being tested by one-way ANOVA. Duncan's multiple range test was used to test the differences between treatment groups. Differences were considered significant at a probability level of 0.05.

TABLE 1.

The changes in body weight (g) of *Penaeus chinensis* during the course of experiment (mean ± SE)[1].

Groups	Initial	At the end of starvation	At different times of recovery growth (day)			
			8	16	24	32
S4	2.201 ± 0.113	2.176 ± 0.111^{ab}	3.245 ± 0.056	4.541 ± 0.229	5.867 ± 0.387	7.212 ± 0.461
S8	2.152 ± 0.021	2.050 ± 0.020^{ab}	3.018 ± 0.045	4.254 ± 0.263	5.656 ± 0.444	7.302 ± 0.732
S12	2.156 ± 0.040	1.966 ± 0.040^{b}	2.851 ± 0.216	3.900 ± 0.263	5.201 ± 0.288	6.594 ± 0.277
C	2.146 ± 0.021	2.146 ± 0.021^{a}	3.074 ± 0.153	4.266 ± 0.302	5.553 ± 0.514	6.846 ± 0.715

[1] Values with different letters in the same column are significantly different from each other ($P < 0.05$).

RESULTS

Growth

The mean body weight changes in the shrimp of the all treatment groups during the course of the experiment are shown in Table 1. During the period of food deprivation all the shrimp in starved groups lost weight and showed characteristic patterns of the mean body weight lost in proportion to the length of starvation periods. At the end of starvation, however, no significant differences in body weight were found among all the experimental groups, except that the shrimp starved for 12 days were significantly lighter than the controls receiving satiation feeding continuously. At the end of refeeding the shrimp previously starved for 4 and 8 days (Groups S4 and S8) were slightly heavier than those fed on full ration throughout, while the most severely starved shrimp were slightly lighter than the controls.

During the course of refeeding the dynamics of SGR_w for all the treatment groups exhibited a similar pattern: the SGR_w tended to decreased with the time of experiment (Fig. 1). There were found to be no significant differences in SGR_w among all groups, either in each 8-day interval of the refeeding period or during the whole course of recovery feeding (Fig. 1; Table 2). By contrast, during the refeeding period, SGR_d, SGR_p, and SGR_c appear to depend on the length of previous starvation periods. It is shown in Table 2 that during the course of refeeding, the SGR_d, SGR_p, and SGR_c did not differ significantly between Group S4 and the control group, but all of these growth rate indexes of Groups S8 and S12 were significantly higher than those of the control.

FI

Changes in feed intake in terms of wet weight (FI_w) for all groups during the refeeding are presented in Figure 2. Compared with the control shrimp, those previously starved shrimp displayed a hyperphagic response to a switch from starvation to satiation feeding. Data in Figure 2 also demonstrate that in the first 8-day interval of refeeding, there were significant differences in FI_w between the starved groups and the control, showing a tendency that the feeding intensities were positively in proportion to the duration of previous food deprivation. However, the appetite of all the starved shrimp dropped rapidly to the level of the controls during the second 8-day of refeeding. Such a case lasted to the end of the experiment, although the most severely starved shrimp showed a slightly higher FI_w than those of the other three groups.

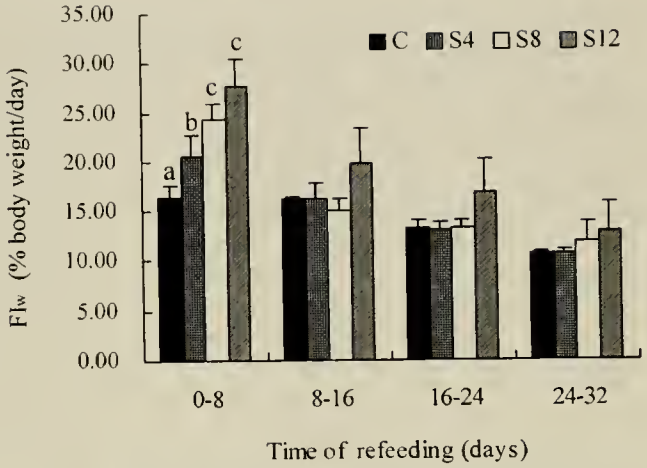

Figure 2. Changes in FI_w of *Penaeus chinensis* during the period of refeeding. Means with different letters within each interval are significantly different ($P < 0.05$) and bars indicate standard errors of the means.

The results on FIs are summarized in Table 2. It is shown that during the course of refeeding, the FIs of Group S12 were significantly higher than those of the other three groups, while the FIs of the other two starved groups were slightly higher than those of the control group.

FCE

Upon realimentation all the previously starved shrimp exhibited a lower FCE_w than that of the controls, with a trend showing that the FCE_w decreased with the duration of the starvation periods (Fig. 3). Different dynamics and extents of restoration in FCE_w of the starved shrimp during the refeeding period are also shown in Figure 3. Within the second 8-day period of refeeding, the shrimp

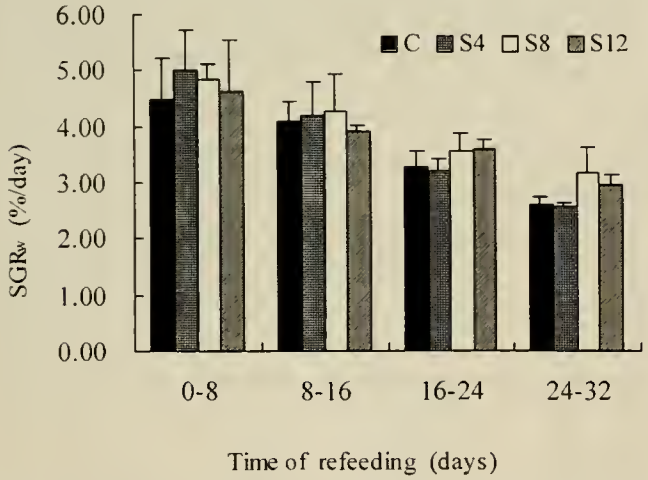

Figure 1. Changes in SGR_w of *Penaeus chinensis* during the period of refeeding. Means with different letters within each interval are significantly different ($P < 0.05$) and bars indicate standard errors of the means.

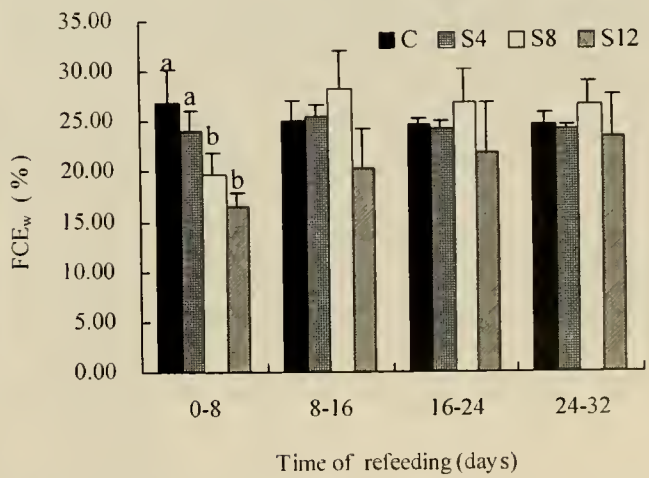

Figure 3. Changes in FCE_w of *Penaeus chinensis* during the period of refeeding. Means with different letters within each interval are significantly different ($P < 0.05$) and bars indicate standard errors of the means.

TABLE 2.

The specific growth rate, feed intake, and food conversion efficiency in *Penaeus chinensis* during the course of experiment (mean ± SE)[1].

Groups	S4	S8	S12	C
SGR_w	3.74 ± 0.06	3.96 ± 0.32	3.78 ± 0.13	3.61 ± 0.35
SGR_d	4.26 ± 0.04[a]	5.15 ± 0.42[b]	5.14 ± 0.25[b]	4.06 ± 0.28[a]
SGR_p	4.25 ± 0.10[a]	5.32 ± 0.32[b]	5.56 ± 0.12[b]	4.05 ± 0.22[a]
SGR_e	4.52 ± 0.13[a]	5.67 ± 0.32[b]	5.70 ± 0.13[b]	4.24 ± 0.25[a]
FI_w	13.66 ± 0.28[a]	13.87 ± 0.83[a]	16.65 ± 2.30[b]	12.93 ± 0.32[a]
FI_d	13.99 ± 0.14[a]	14.04 ± 0.99[a]	17.39 ± 3.07[b]	12.67 ± 0.29[a]
FI_p	14.36 ± 0.30[a]	14.52 ± 0.90[a]	17.86 ± 2.53[b]	13.37 ± 0.30[a]
FI_e	15.70 ± 0.33[a]	15.36 ± 0.96[a]	19.63 ± 2.78[b]	13.65 ± 0.29[a]
FCE_w	24.55 ± 0.78	25.31 ± 2.34	20.53 ± 3.37	25.14 ± 1.37
FCE_d	26.47 ± 0.43	30.26 ± 3.86	24.76 ± 3.68	28.13 ± 1.58
FCE_p	25.77 ± 0.78	29.83 ± 2.37	25.13 ± 3.89	26.65 ± 1.21
FCE_e	24.66 ± 0.73	29.33 ± 2.27	23.25 ± 3.60	26.98 ± 1.14

[1] Values with different letters in the same row are significantly different from each other ($P < 0.05$). SGR_w, SGR_d, SGR_p, and SGR_e: specific growth rates expressed in terms of wet weight, dry matter, protein, and energy content (%/day). FI_w, FI_d, FI_p, and FI_e: feed intake expressed in terms of wet weight, dry matter, protein, and energy content (%/day). FCE_w, FCE_d, FCE_p, and FCE_e: food conversion efficiency expressed in terms of wet weight, dry matter, protein, and energy content (%).

previously starved for 4 days rapidly recover their FCE_w to the level of the controls, while those starved for 8 days displayed an improved FCE_w slightly higher than the controls. This pattern of restoration for the two starved groups was maintained to the end of refeeding period. In contrast, the most severely starved shrimp (Group S12) took a longer period (24 days) to restore the FCE_w approximate to that of the controls.

During the whole course of refeeding, FCEs did not significantly differ among all groups, and it seemed that the shrimp previously starved for 8 days displayed slightly improved FCEs compared with the controls.

Chemical Composition and Energy Content

Chemical composition and energy content of the food (polychaete worms) were determined to be as follows: moisture was 74.74%, lipid was 2.37%, protein was 19.36%, ash was 1.66%, and energy content was 5.424 kJ/g wet weight.

Data on body composition and energy content are presented in Table 3. At the end of food deprivation water content tended to increase, while lipid, protein, and energy content decreased with the prolongation of the starvation periods. Ash content was not significantly affected by starvation. At the end of refeeding period there were no significant differences in water, protein, ash, and energy content between the starved and control groups, except the case that lipid content was still lower both in Group S4 and S12 than that in the control group.

DISCUSSION

Previous studies of compensatory growth have shown the ability to elicit above normal growth rates in fish and other animals (Wilson and Osbourn 1960, Russell and Wootton 1992). The results for rainbow trout, *Oncorhynchus mykiss* (Walbaum) reported by Quinton and Blake (1990) showed that the fish fed on the "3 weeks starvation and 3 weeks feeding" cycle out-performed the control group and that it was during the last week of refeeding that the great increase in growth associated with compensatory growth response occurred. The work by Miglavs and Jobling (1989b) on juvenile Arctic charr, *Salvelinus alpinus* (Linnaeus) indicated that shortly after transfer from a restricted to a satiation feeding regime, growth rates of the fish increased markedly and were significantly higher than those of the control. In the present study the previously food-deprived shrimp did not display significantly higher specific

TABLE 3.

The chemical composition and energy content in *Penaeus chinensis* at different times of the experiment in relation to duration of starvation in the food deprivation period (mean ± SE)[1].

Time	Groups	Water[2]	Protein[2]	Lipid[2]	Ash[2]	Energy[3]
At the end of starvation	S4	78.33 ± 0.67[a]	16.20 ± 0.54[a]	1.02 ± 0.02[b]	2.83 ± 0.18	3.876 ± 0.136[b]
	S8	81.70 ± 0.53[b]	12.99 ± 0.66[b]	0.86 ± 0.03[c]	2.95 ± 0.17	3.125 ± 0.196[c]
	S12	82.92 ± 1.06[b]	11.38 ± 0.75[b]	0.86 ± 0.07[c]	3.12 ± 0.23	2.784 ± 0.205[c]
	C	76.92 ± 0.82[a]	16.79 ± 0.68[a]	1.46 ± 0.05[a]	2.87 ± 0.09	4.394 ± 0.118[a]
At the end of recovery growth	S4	74.43 ± 0.50	19.08 ± 0.39	1.77 ± 0.05[b]	2.79 ± 0.08	4.975 ± 0.088
	S8	73.15 ± 1.74	20.04 ± 1.24	2.53 ± 0.16[a]	2.73 ± 0.26	5.395 ± 0.372
	S12	73.60 ± 1.13	20.03 ± 0.88	1.66 ± 0.08[b]	2.78 ± 0.22	5.143 ± 0.247
	C	73.37 ± 0.64	19.34 ± 0.55	2.55 ± 0.12[a]	2.60 ± 0.14	5.371 ± 0.121

[1] Values with different letters in the same column are significantly different from each other ($P < 0.05$).

[2] Values were expressed as the percentage of wet weight.

[3] Values were expressed as kJ per gram of wet weight.

growth rate in terms of wet body weight (SGR_w) than the controls during any of the 8-day intervals of refeeding, but this cannot preclude the fact that the compensatory growth response occurred in Chinese shrimp. As Jobling (1994) pointed out, "within the framework of the energy balance equation, growth is defined as an increase in the energy content of the fish body," which means that growth is dependent on two aspects, wet body weight and body composition of the fish. The results of the present study showed that SGR in terms of dry matter, protein, and energy content (SGR_d, SGR_p, and SGR_e) of the shrimp previously starved more than 8 days were significantly higher than that of the control during the course of refeeding (Table 2). This obviously indicates that Chinese shrimp displayed compensatory growth response when changed from starvation to satiation feeding.

Wieser et al. (1992) reported that the growth rate after refeeding increased in proportion to the length of starvation periods in three cyprinid species. Similar results for the European minnow, *Phoxinus phoxinus* (Linnaeus) were observed by Russell and Wootton (1992) and for *Procambarus clarkii* (Girard) by Bostworth and Wolters (1995). The results of this study appear to be consistent with the above findings in which the strength of compensatory growth response depends on the length of the starvation periods. Table 2 shows that during the course of refeeding, the shrimp previously subjected to food deprivation for 4 days just displayed a slight compensatory growth response, while those previously starved for more than 8 days showed noticeable compensatory growth responses.

In agreement with previous studies performed on fish and domestic animals (Wilson and Osbourn 1960, Russell and Wootton 1992, Hayward et al. 1997), Chinese shrimp also responded to a switch from food deprivation to satiation feeding by exhibiting hyperphagia. The extent of the hyperphagic response generally depends on two variables, namely the feeding intensity and the duration of appetite elevation. There are two patterns of appetite dynamics following various periods of food deprivation: The duration of hyperphagia is similar for the animal starved for different periods, but the feeding intensity increases in proportion to the length of starvation periods (Russell and Wootton 1992), and the initial appetite is similar, but the duration of hyperphagia is variable (Russell and Wootton 1993, Bull and Metcalfe 1997). The results of the current experiment indicated that the feeding intensity increased with the length of starvation periods, but the hyperphagic response in all starved groups just occurred within the first 8-day interval of refeeding (Fig. 2), which appear to provide an evidence for the first pattern of appetite dynamic. The above findings implied that the patterns of appetite dynamics seem to be dependent on animal species.

Improved food conversion efficiency is also an aspect frequently highlighted in studies of compensatory growth and may be of practical implications in the production of fish and domestic animals (Wilson and Osbourn 1960; Dobson and Holmes 1984, Quinton and Blake 1990). The evidence for the improved food conversion efficiency may be caused by the reduced basal metabolism occurred during starvation extending into the initial stage of realimentation (Boyle et al. 1981, Yambayamba et al. 1996) or by the differences in the composition of weight gain between animals displaying compensatory growth and those fed continuously, the former often tending to deposit fewer proportions of the gain as body fat (Yu et al. 1990, Jobling et al. 1994). In the present study there were no significant differences in food conversion efficiencies between the shrimp previously starved for various periods and the controls during the course of refeeding, with the exception of the case that Group S8 showed slightly higher food conversion efficiencies in terms of dry matter, protein, and energy content (FCE_d, FCE_p, and FCE_e) than those of the control group (Table 2). Furthermore, Figure 3 clearly depicts that during the first 8 days of refeeding the previously starved shrimp displayed lower food conversion efficiency in terms of wet weight (FCE_w) than the controls, and that the longer the shrimp remained without feeding, the lower the FCE_w was. The results of this study based on the analyzing of the changes in body composition and the FCE_d, FCE_p, and FCE_e during the course of refeeding seem to provide an evidence that the lower FCE_w may be attributable to the changes in body water content. In other words, if the same amount of dry matter, protein, or energy content was synthesized, the starved shrimp will relatively absorb less amount of water than the controls during the first 8 days of refeeding.

In fish (Miglavs and Jobling 1989a, Wang et al. 1999) and other crustaceans (Barclay et al. 1983, Stuck et al. 1996), starvation generally leads to an increase in water content and to reductions in lipid, protein, and energy content. A similar pattern was observed in the current study. There was a trend for the shrimp that the extent of increase in water content and decreases in lipid, protein, and energy content depends on the length of starvation periods (Table 3). There were different conclusions drawn from the previous studies on the order of utilization of major energy reserves in crustaceans (reviewed by Whyte et al. 1986). In the present study the results calculated from the data of Tables 1 and 2 show that protein contributed 24.84% and lipid contributed 31.68% of total metabolized energy during 4 days of starvation, while in 12 days of starvation, protein contributed 62.49% and lipid contributed 14.45%, which was in agreement with the suggestion by Cuzon et al. (1980) and Barclay et al. (1983) that protein was the major source of energy used during prolonged starvation. After 32 days of refeeding there were no significant differences in water, protein, and energy content between the starvation-satiation shrimp and the controls, except that lipid content in the shrimp previously subjected to starvation for 4 or 12 days was still significantly lower than that of the controls (no obvious explanation on this case is available). This indicates that Chinese shrimp have the ability to withstand and recover from relatively prolonged starvation.

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THE USE OF POULTRY MORTALITIES AS AN ALTERNATIVE BAIT FOR THE HARVESTING OF BLUE CRABS *CALLINECTES SAPIDUS* (RATHBUN, 1885)

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ABSTRACT Alternative bait products for the harvesting of blue crabs are needed because traditional baits are becoming increasingly expensive and more difficult to acquire. Poultry mortality carcasses and poultry mortality silage were compared to menhaden for their ability to attract blue crabs using on-shore attractant trays as well as off-shore crabpot protocols. When product stability in water was eliminated as a variable in a preliminary investigation, there was no significant difference ($P > .10$) in the total number of blue crabs harvested using the alternative poultry silage baits versus a menhaden bait control. Attractant tray evaluations indicated an increased preference ($P < .035$) of female crabs *versus* male crabs for the alternative poultry baits relative to a menhaden control but failed to demonstrate any significant differences ($P > .152$) in the preferences of blue crabs for the pH, binder, betaine addition, or poultry meat form used in the alternative bait formulations. In contrast, a marine evaluation of the alternative bait treatments indicated an increased preference ($P < .005$) for poultry bait products containing no supplemental betaine and a decreased preference ($P < .045$) for poultry baits adjusted to a pH = 8. A consumer sensory evaluation of the crabmeat harvested from crabs with the alternative baits demonstrated that no off flavors are associated with the use of the alternative poultry baits. Properly formulated, poultry mortality could be utilized as alternative bait for the harvesting of blue crabs and has potential for use as bait for other aquatic species.

KEY WORDS: blue crab, poultry mortality, alternative bait products

INTRODUCTION

The blue crab fishery is the largest commercial fishing industry in the United States, with average landings exceeding 95,000 metric tons per year. (United States Department of Commerce 1995). Dockside values for the blue crab fishery are second only to the snow crab, with 1994 dockside values exceeding $137 million (USDC 1995).

Harvesting of blue crabs occurs mainly by trapping in baited wire cages known as crabpots. Individual blue crab fishermen generally work some 200–300 crabpots set across inland bays, rivers, and estuaries. Millions of pounds of fish by-catch, river herring, and menhaden are used annually to harvest the blue crabs. Unfortunately, these traditional bait products are becoming increasingly expensive and more difficult to acquire. Mandatory use of by-catch reduction devices, recent net bans, and an increased demand for menhaden for fish meal production have increased the prices for traditional baits and forced watermen to use less-effective products as alternative baits (e.g., shrimp heads, gar, and gizzard shad). The overall cost of bait for the harvesting of blue crabs has risen more than 300% in the previous 5 y (Johnson et al. 1996). Continuing prosperity of this major coastal business requires that a plentiful, cost-competitive bait supply be available.

In an effort to develop alternative bait products to meet the demands of this industry, many researchers have investigated compounds that elicit positive responses in the chemoreceptor organs of various aquatic crustaceans (Laverack 1963, Levandowsky and Hodgeson 1965, Lenhoff and Lindstedt 1974, Zimmer–Faust 1987, Rittschof 1992). Since 1897, it has been known that chemoreception plays an important role in the food-seeking behavior of crabs (Bethe 1897). Detection occurs from a distance, so it is evident that a highly soluble chemical is carried by water currents and is detected by the crustacea (Laverack 1963). Substances commonly present in crustacean foods that might readily leach out of damaged tissues are trimethyl amine oxide in fish and betaine in invertebrates (Laverack 1963). Unfortunately, previous efforts to identify compounds as alternatives to traditional baits have met with limited success. The attractant qualities of baits cannot be totally accounted for by any one major component extracted from natural baits (Shelton and Mackie 1971). There is a need for complex mixtures of compounds in specific concentration ratios to mimic the stimulatory effect of whole tissue (Mackie 1982).

Poultry mortality silage is a biosecure, semi-solid product resulting from lactic acid fermentation of ground poultry carcasses

(Murphy and Silbert 1990, Cai and Sander 1995). While the acidic silage product was not accepted by the blue crabs, preliminary test results indicated that neutralized silage products were readily detected in the aquatic environment and rapidly consumed by this species. Therefore, once pH adjusted, this material does appear to contain the stimulatory components in the proper ratios necessary to attract the crabs to the bait (Middleton and Hines, unpubl. data). The objective of this research was to evaluate the effectiveness of poultry mortality silage as alternative bait for the harvesting of blue crabs. The use of this material as a bait could reduce the demands placed on our rivers and estuaries by the mass harvesting of bait fish and provide an economical, environmentally friendly alternative bait source for the crabbing industry. In addition, a major recycling/reutilization outlet for poultry mortality will have been developed.

MATERIALS AND METHODS

Bait Manufacture

Preliminary Evaluation

A preliminary evaluation was conducted to evaluate poultry mortality silage as a potential alternative bait for the harvesting of blue crabs. Turkey mortality was deplumed, ground, and stabilized by lactic acid fermentation for 4 wk to produce a silage (Murphy and Silbert 1990, Blake et al 1992, Cai and Sander 1995). Bait quality menhaden was obtained locally (Craven Crab Company, New Bern, NC 28560). Immediately prior to bait manufacture, the poultry silage was neutralized using solid sodium hydroxide (Fisher Scientific, Fairlawn, NJ 07410). Anhydrous betaine (Betafin BT™, Finnsugar Bioproducts, Naantali, Finland) was added at 150 mg/100 g silage material to one half of the neutralized poultry silage. To eliminate variation due to structural stability, all bait products were prepared by grinding in a commercial meat grinder (Model A-200, Hobart Mfg. Co., Troy, OH 45373) and gelled using a sodium alginate binder (Keltone HV®, Nutrasweet Kelco Co., Chicago, IL 60661) according to manufacturer's recommendations. Calcium sulfate hemihydrate (Plaster of Paris) was used as the source of calcium ions (Fisher Scientific, Fairlawn, NJ 07410). Bait products were pressed into 1.5-inch collagen sausage casings (Coria®, Devro Teepak®, Summerville, SC 29483) and refrigerated at 5 °C for 12 h to allow for solidification of the bait material. Baits were then individually packaged in polyethylene storage bags (Ziplock Freezer Bags, Dowbrand, L. P., Indianapolis, IN 46268) and frozen at −20 °C until used.

Attraction Tray Trial 1

A 2 × 4 × 4 factorial design was used to evaluate the different experimental bait treatments that included: two poultry meat forms (fresh frozen *versus* fermented); four pH levels (5.5, 7.5, 9.5, and 11.5); and four binding agents (Keltone HV®, Mannugel®, wheat gluten with soy protein, and Gelcarin ME9111). Poultry mortality silage, prepared by lactic acid fermentation (Murphy and Silbert 1990, Blake et al. 1992, Cai and Sander 1995), and frozen ground poultry mortality, both from Ross 308 43-day-old male broilers (Ross Breeders, Inc. Huntsville, AL 35805) were obtained as raw materials for poultry bait manufacture. Aliquots of the raw materials were adjusted with solid sodium hydroxide (Fisher Scientific, Fairlawn, NJ 07410) to a pH of 5.5, 7.5, 9.5, or 11.5. Each of these eight treatments were then formed according to supplier's recommendations into bait products using either Keltone HV® (Nutrasweet Kelco Co, Chicago, IL 60661), Mannugel® (Nutrasweet Kelco Co, Chicago, IL 60661), Gelcarin ME 9111 (FMC Corp., Philadelphia, PA 19103), or 3% wheat gluten and 3% soy protein (Vital Wheat Gluten, Midwest Grain Products, Inc., Atchison, KS 66002 and Promine DS, Central Soya Company, Inc., Fort Wayne, IN 46802) as binding agents. Encapsulated calcium lactate pentahydrate (Cap-shure®, Balchem Corp., Slate Hill, NY 10973) was used as a source of calcium ions to cold set the sodium alginate binders (Keltone HV® and Mannugel®). The latter two binding systems were heat set in 140 °C ovens to an internal temperature of 85 °C. Molds were utilized to manufacture 100-g replicates of each bait treatment. Replicates were refrigerated at 5 °C for 12 h to allow for solidification of the bait products. One hundred-gram fillets were prepared for use as control baits from locally obtained bait quality menhaden (Craven Crab Co., New Bern, NC 28560). Baits were then individually packaged in polyethylene storage bags (Fisher Scientific, Fairlawn, NJ 07410) and frozen at −20 °C until utilized.

Attraction Tray Trial 2

A 2 × 2 × 4 factorial design was used to evaluate the different experimental bait treatments that included: two poultry meat forms (fresh frozen *versus* fermented); the presence or absence of betaine in the bait formulation; and four pH levels (6, 7, 8, and 9). Raw materials for bait manufacture were mortality silages prepared by lactic acid fermentation (Murphy and Silbert 1990, Blake et al. 1992, Cai and Sander 1995) or frozen ground poultry mortality, both from 25-week-old Arbor Acres Yield male broiler breeders (Arbor Acres Inc., Glastonbury, CT 06033). Anhydrous Betaine (Sigma Chemical Co, St. Louis MO 63178) was added at 250 mg/100g silage (250 mg%) to one-half of the fresh as well as the silage material. Aliquots of each of the four treatments were pH adjusted with solid sodium hydroxide (Fisher Scientific, Fairlawn, NJ 07410) to a pH of 6, 7, 8, or 9. Cheesecloth squares were used to contain 100-g replicates of each bait treatment. One hundred-gram fillets were prepared for use as control baits from locally harvested bait quality menhaden. Baits were then individually packaged in polyethylene storage bags (Fisher Scientific, Fairlawn, NJ 07410) and frozen at −20 °C until utilized.

Ocean Evaluation

Poultry bait treatments were prepared from raw materials as used in the second attraction tray study and gelled into bait products using Gelcarin ME 9111 (FMC Corp., Philadelphia, PA 19103). To increase structural stability of the poultry baits, treatments were pressed into 1.5-inch diameter clear fibrous sausage casings with three 0.44 mm diameter holes per square inch (Vista International Packaging, Inc. Kenosha, WI 53141) to produce sausage shaped bait products averaging 350 g. Poultry bait sausage products were heated in a 140 °C oven to an internal temperature of 85 °C and then refrigerated at 5 °C for 12 h to allow for solidification. Bait quality menhaden fish were harvested locally. Baits were individually packaged into polyethylene storage bags (Ziplock Freezer Bags, Dowbrands L. P., Indianapolis, IN 46268) and frozen at −20 °C until used.

Consumer Sensory Panel Evaluation

Mortality silage prepared by lactic acid fermentation (Murphy and Silbert 1990, Blake et al. 1992, Cai and Sander 1995) and frozen ground poultry mortality, both from 25-wk-old Arbor Acres Yield male broiler breeders (Arbor Acres Inc., Glastonbury, CT 06033) were obtained as raw materials for bait manufacture. The poultry products were adjusted to pH = 7.5 using solid sodium

hydroxide (Fisher Scientific, Fairlawn, NJ 07410) and gelled into 400-g bait products using Gelcarin ME 9111 (FMC Corporation, Philadelphia, PA 19103). Bait products were heated in a 140 °C oven in glass beakers used as molds to an internal temperature of 85 °C and allowed to set under refrigeration for 12 h at 5 °C. Bait quality menhaden were harvested locally. Individual baits were stored in polyethylene storage bags (Fisher Scientific, Fairlawn, NJ 07410) and frozen at −20 °C until used.

Experimental Design

Preliminary Evaluation

The effectiveness of each silage bait product (as measured by the number of crabs harvested/pot/day) was compared to that of the bait product manufactured from menhaden fish. Empty pots were used as negative controls. Fifteen four-funnel crabpots were baited and harvested daily for 11 days (September 24 through October 4, 1997) in Pettiford Creek, located in Carteret County North Carolina. Baits and negative controls were randomly assigned and rotated every 3 days. The number of blue crabs/pot as well as sex determination information was recorded for each day's harvest.

Attraction Tray Evaluations

Attraction trays were constructed similar to the mazes developed by Shelton and Mackie (1971) to study the feeding preferences of the shore crab [*Carcinus maenas* (L.)], but appropriately scaled for the larger size and greater motility of the blue crab (Fig. 1). The trays consisted of three chambers, a large main chamber and two smaller test chambers that open into the main chamber by entrances with small ramps. These ramps permitted the crabs to enter the test chambers if attracted by the bait product, but restricted them from leaving. Entrances to the test chambers were equipped with sliding perforated partitions that restricted crab entry to the test chambers until permitted to do so by the experimenter. Each test chamber contained a small perforated bait compartment through which incoming water flowed from a common tank. The outlet flow from the trays was through an overflow pipe set in the main chamber to a height of four inches. In contrast to the multiple crab testing protocol of Shelton and Mackie (1971), aggressive behavioral characteristics of the blue crabs required that the responses to the various baits be evaluated using only one blue crab per trial. Therefore, the protocol proposed by Shelton and Mackie (1971) was modified to compare test bait products to menhaden fish fillets rather than to seawater as the control. The attractiveness of the various bait preparations relative to the fish fillets was evaluated by chi-squared protocols to reflect a positive-negative outcome of the individual crabs to the test bait products or the fish fillet controls.

Husbandry practices similar to those employed in crab shedding operations were used during testing protocols. Male and female crabs were separated and held for a minimum of 3 days prior to testing to allow acclimation to captivity and to decrease re-

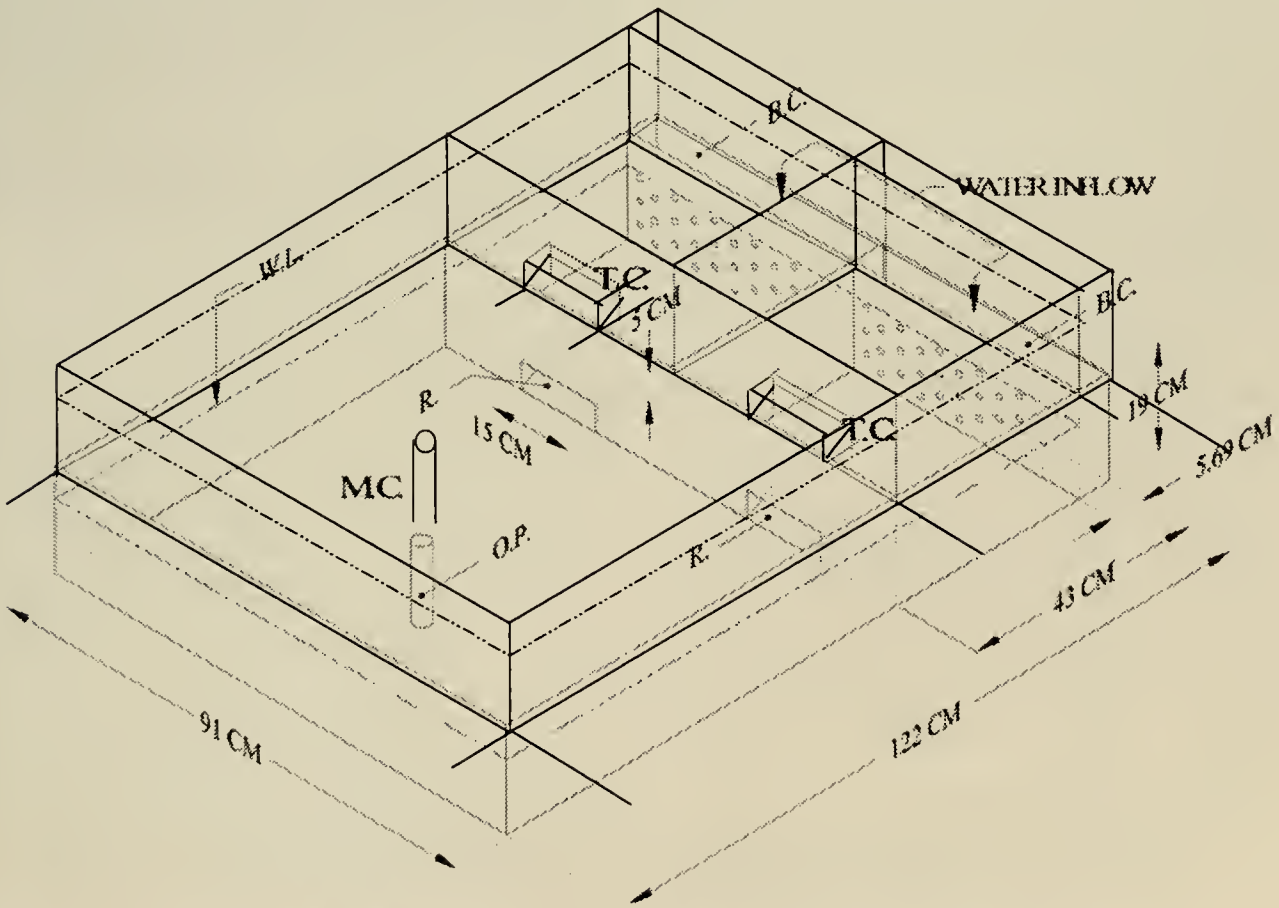

Figure 1. Attraction tray design. M.C., Main Chamber; T.C., Test Chambers; B.C. Bait Compartment; O.P., Overflow Pipe; R., Ramp at entrance to test chamber; W.L., Water Level. Design modified from experimental maze of Shelton and Mackie, 1971. (Drawing courtesy Hammond/Vaughan, Inc. Cad Designs, Garner, NC 27529).

sponse times to bait. Any crabs exhibiting signs of molting were removed from the holding tanks. Water temperatures of between 23.9 and 29.4 °C with dissolved oxygen levels of greater than 4 mg O_2/L were maintained in the holding tanks as well as the water flowing through the attraction trays. Flow rates were kept constant and equal in each test chamber of the attraction tray and water levels were greater than 4 inches at all times.

Individual crabs were placed in the main chamber of the baited attraction trays near the outflow pipe and allowed to acclimate until escape behaviors moderated. When acclimation was complete, the partitions between the large main chamber and the smaller test chambers were removed in a manner that did not disturb the subject crab. Crabs could then move, depending on crab preferences, into the chamber containing the test or control bait (fish fillet). Test and control baits were utilized for five replicate trials with good stimulatory responses. Response was recorded and subsequent trials initiated. Test and control baits were randomly assigned to the test chambers and chambers were thoroughly cleaned and flushed with fresh water prior to the initiation of subsequent trials.

Trial One

A 2 × 2 × 4 × 4 factorial design was employed to evaluate the various bait formulations. Male and female crabs were each used to evaluate the preferences of blue crabs for baits manufactured using the two poultry meat forms, the four different pH levels, and the four different binding agents. Two replicate sets of five male and five female crabs were used to evaluate each of the bait formulations. The probability of choice relative to 100-g menhaden fillets (Craven Crab Company, New Bern, NC 28560) was determined for each product.

Trial Two

A 2 × 2 × 2 × 4 factorial design was used to further evaluate the various alternative bait formulation possibilities. Male and female crabs were each used to evaluate the preferences of blue crabs for baits manufactured using the two poultry meat forms, the presence or absence of betaine, and the four pH levels. Three replicate sets of five crabs each were used for each bait formulation for both male and female crabs. The probability of choice relative to locally harvested menhaden fillets was calculated as in Trial One.

Ocean Evaluation

Each of the poultry bait products, as well as the menhaden fish control utilized during the second attraction tray trial, was evaluated for its ability to attract blue crabs in their natural environment using traditional four-funnel crab pots. Two replicate crabpots of each bait treatment or treatment combination were set for six 48-h harvesting periods (November 7 through November 19 1998) in Pettiford Creek, Carteret County NC. The number of male and female crabs harvested using each bait product per pot per day was recorded.

Consumer Sensory Panel Evaluation

Female blue crabs were held for a period of 5 days in traditional crab shedding tables and allowed access to either a control bait of locally harvested menhaden, or an alternative bait product manufactured from either freshly frozen poultry mortality or poultry mortality stabilized by lactic acid fermentation. Crabs were then cooked and cleaned under commercial conditions (Luther Lewis and Son Crab Co., Davis, NC 28524) and lump and backfin crabmeat was combined and stored in polyethylene storage bags (Ziplock Freezer Bags, Dowbrands L. P., Indianapolis, IN 46268) for 14 h at 5 °C pending consumer sensory panel evaluation.

Immediately prior to the initiation of the sensory panel evaluation, 12-g portions of lump and backfin crabmeat were placed in preheated glass baby food jars set in preheated sand, and maintained in a 375 °F convection oven for 15 min. The consumer panel consisted of 31 individuals chosen from the Departments of Food Science and Poultry Science, North Carolina State University, Raleigh, NC. Panelists evaluated the degree of differences in aroma, flavor and texture between crabmeat from crabs consuming the menhaden control and crabmeat from crabs that consumed the alternate bait treatments using a seven-point scale. Samples were presented simultaneously in a balanced, random order from each bait treatment group with a blind control included as one of the samples. Panelists worked in individual booths equipped with red lights and no discussion took place during the evaluation. Randomization of the order of presentation was used to control for contrast and carry-over effects of the various samples (Meilgaard et al. 1991).

Statistical Analysis

All data obtained during the taste panel as well as the preliminary and ocean evaluations were tested for significance using the General Linear Model (GLM) procedures of SAS (SAS Institute, 1996). Treatment effects were considered significant at $P < .05$. The LSMeans procedure of SAS was used to determine significant differences among treatments. Attraction tray data were tested for significance by chi-squared analysis using the GENMOD procedures of SAS (SAS Institute, 1996). Probability of choice was calculated using the formula: $X = \ln (P/1 - P)$, where X = Genmod probit value estimate (Genmod device for the unrestricted calculation of probabilities) and P = probability of choice. Dunnett's T means procedures of SAS were also employed for the taste panel data to compare each treatment mean to the control.

RESULTS AND DISCUSSION

Preliminary Evaluation

Highly significant ($P < .001$) bait effects were observed in the harvest values (Table 1). The crabs were significantly more attracted to all bait products than to the negative controls ($P < .04$). Similar preferences ($P > .05$) were demonstrated by the female crabs for the poultry silage baits in comparison to the menhaden fish bait. However, they were significantly less attracted ($P < .035$) to the poultry silage bait when betaine was included in the bait formulation. Male crabs demonstrated a significant preference ($P < .034$) for the menhaden bait over that of either poultry silage preparation. However, when total blue crab harvest values were analyzed, there were no significant differences ($P > 0.10$) in the total number of blue crabs harvested using the poultry silage bait versus the menhaden bait. The addition of betaine to the poultry silage bait did not significantly ($P > 0.56$) affect total harvest values when compared to the poultry silage alone. The increased number of male crabs harvested during this trial is presumably indicative of the population dynamics of the area surveyed during the evaluation and is not likely a reflection of bait treatments.

Attraction Tray Trial I

There was a significantly ($P < .035$) greater probability of preference for the poultry bait relative to fish demonstrated by female crabs than by male crabs; in agreement with the results of

TABLE 1.

Average daily blue crab harvest[a]: preliminary evaluation.

Treatment	Male Crabs	Female Crabs	Total Crabs
Negative control (empty pot)	1.333[3]	.1212[3]	1.454[3]
Menhaden bait	3.659[1]	.6818[1,2]	4.341[1]
Poultry silage (PS) bait	2.727[2]	.909[1]	3.636[1,2]
PS + betaine bait	2.864[2]	.5227[2]	3.386[2]
Statistics (pooled)			
Treatment effects (*P* values)	.001	.001	.001
SEM[b]	.303	.148	.349

[1–3] Means within columns with no common superscript differ significantly ($P < .05$).

[a] Average number of blue crabs harvested/pot/day.

[b] SEM = Standard Error of the Mean with 121 degrees of freedom.

the preliminary evaluation (Table 2). No significant differences were found in the probability of choice for pH, binder, or meat form used in the bait treatments ($P > .180$). No significant two-way factor interactions were demonstrated ($P > .0751$). Significant three-way interactions were demonstrated for pH*binder*meat and pH*meat*sex ($P < .010$ and $P < .039$, respectively); however, nothing meaningful was discerned by the evaluation of these interaction patterns. The poultry bait was chosen consistently less often than the fish controls by both sexes of crab throughout the experiment. The probability of choice of poultry bait product versus menhaden fillet was $\geq 48.65\%$.

Attraction Tray Trial 2

No significant differences ($P > .152$) or interactions ($P > .085$) in any of the parameters were demonstrated relative to sex, pH, meat form, or the addition of betaine (Table 3). The poultry baits were chosen consistently more often than the fish fillet controls throughout the course of this experiment. The probability of choice of poultry bait product *versus* menhaden fish fillet was $\leq 66.72\%$.

TABLE 2.

Probability of choice by blue crabs of poultry bait formulations relative to menhaden in attraction tray trial[a].

Factor Category	Factor	Probability of Choice[b,c]	*P* Values[c]
Sex	Male	40.18%[2]	.0346
	Female	48.65%[1]	
Binder	Keltone HV	51.84%	.1807
	Mannugel	40.88%	
	Soy/Wheat	42.60%	
	Gelcarin	48.65%	
Meat	Fresh	51.15%	.5402
	Fermented	48.65%	
pH	5.5	42.78%	.1982
	7.5	53.75%	
	9.5	43.86%	
	11.15	48.65%	

[1–3] Means within factor categories with no common superscript differ significantly ($P < .05$).

[a] Probability of choice of treatment main effects. No significant or meaningful two-way or three-way treatment interactions demonstrated.

[b] Probability of choice of poultry bait formulation relative to menhaden fish fillets.

[c] Probability of choice (P) was calculated using the formula: $X = \ln(P/1 - P)$, whereas X = Gemmod probit value estimate.

[d] P values were determined by χ^2-squared analysis using the Genmod Procedures of SAS (SAS Institute, 1996).

Ocean Evaluation

Difficulties were encountered in this trial due to the casings utilized to form set the bait products. Although the casings were manufactured with three 0.44 mm pinpoint openings per square inch to allow flavor compounds from the mortality silage contents to readily disperse into the seawater, these openings apparently were sealed during the cooling process and impeded flavor release during the ocean evaluation. In order to attract blue crabs, the casings of the alternative bait products were slit repeatedly to facilitate seawater contact with internal contents for flavor compound release. Therefore, the available attractant surface area in the alternative bait products was limited to areas exposed by cutting the casings. While the attractant surface areas among the alternative bait products were comparable (and therefore comparisons between the attractant qualities of alternative products would be valid), the attractant surface area of the alternative bait products relative to the traditional menhaden fish bait was compromised. Physical stability of the alternative bait products in water was demonstrated to be ≤ 5 days; therefore, casing materials on poultry bait products were unnecessary, resulted in reduced harvest yields, and are to be avoided in future trials.

The number of blue crabs harvested from the ocean using traditional menhaden fish as bait was significantly greater ($P < .001$) than the number of crabs harvested using either of the alternative poultry bait products (Table 4). There was no significant difference ($P > .381$) in the average number of blue crabs harvested between the fresh and fermented alternative bait products. A significantly greater ($P < .001$) number of female crabs were attracted to the poultry bait than were male crabs (Table 5). However, a significantly larger ($P < .003$) number of female crabs were also attracted

TABLE 3.

Probability of choice by blue crabs of poultry bait formulations relative to menhaden in attraction tray trial 2[a].

Factor Category	Factor	Probability of Choice[b,c]	*P* Value[d]
Sex	Male	66.72%	.7737
	Female	67.93%	
pH	6	58.60%	.1995
	7	55.90%	
	8	57.59%	
	9	67.93%	
Meat	Fresh	61.60%	.1525
	Femented	67.93%	
Betaine	No	67.09%	.8449
	Yes	67.93%	

[a] Probability of choice of treatment main effects. No significant ($p < .085$) two-way or three-way treatment interactions demonstrated.

[b] Probability of choice of poultry bait formulation relative to menhaden fish fillets.

[c] Probability of choice (p) was calculated using the formula: $X = \ln(P/1 - P)$, where X = Genmod probit value estimate.

[d] P values were determined by χ^2-squared analysis using the Genmod Procedures of SAS (SAS Institute, 1996).

TABLE 4.

Average blue crab harvest in ocean evaluation[a].

Bait Product	Male Crabs	Female Crabs	Total Crabs
Fermented poultry bait	1.187[1]	2.292[2]	3.479[2]
Fresh poultry bait	1.375[1]	2.365[2]	3.739[2]
Fish bait	3.979[2]	8.104[1]	12.083[1]
Statistics (pooled)			
Treatment effects (P values)	.001	.001	.001
SEM[b]	.416	.625	.490

[1–2] Means within columns with no common superscript differ significantly ($P < .05$).
[a] Average number of blue crabs harvested/pot/day.
[b] SEM = Standard Error of the Mean with 237 degrees of freedom.

to the fish bait products. Therefore, the sex preferences demonstrated in the previous trials cannot be confirmed by these data due to the larger population of female crabs in the survey area.

When alternative poultry bait formulations were compared, significant differences were demonstrated in the preferences of blue crabs for specific pHs of bait products as well as the inclusion or exclusion of betaine in the formulation. While no linear regression trend was established for pH ($P > .05$), significant differences ($P < .045$) were demonstrated in the total number of blue crabs harvested with the various pH formulations, with a reduced preference indicated for baits of pH = 8 (Table 6). In addition, baits that did not contain betaine were significantly ($P < .005$) more attractive to the crabs than were bait products containing the betaine supplementation (Table 7). No significant differences ($P > .090$) were demonstrated in either of these parameters when the blue crab harvest was analyzed by sex (data not shown). No two-way or three-way treatment interactions were demonstrated among the bait treatment formulations for male, female or total crabs harvested ($P > .174$).

This research failed to demonstrate that attractant trays are effective in predicting harvest value tendencies for blue crabs in their natural environment. Ocean testing is capable of detecting significant bait formulation preferences of blue crabs that were not differentiated ($P > .05$) using the attractant tray protocol (Table 8). Modifications of the attractant tray design sample size alterations, and/or additional replicates of each treatment evaluated might improve the predictive nature of this alternative protocol. Additional research with corresponding ocean testing is required.

Consumer Sensory Panel Evaluation

No significant differences ($P > .242$) were demonstrated in the aroma, flavor, or texture of lump and backfin crabmeat harvested from crabs consuming the various bait products (Table 9). When direct one-on-one comparisons were made (Dunnett's T means procedures of SAS), no significant differences ($P > .05$) were demonstrated in these parameters between crabmeat harvested from crabs consuming either of the alternative bait products and those consuming traditional menhaden fish (data not shown).

CONCLUSION

Poultry mortality silage has demonstrated potential as alternative bait for the harvesting of blue crabs. When given a choice between fish and a poultry bait product (in the attractant tray),

TABLE 5.

Effect of crab sex on combined harvest: ocean evaluation[a].

Category of Crab	Poultry Bait	Fish Bait
Male	1.281[2]	3.979[2]
Female	2.329[1]	8.104[1]
Statistics (pooled)		
Treatment effects (P values)	.001	.003
SEM[b]	.179	.949

[1–2] Means within columns with no common superscript differ significantly ($P < .05$).
[a] Average number of blue crabs harvested/pot/day.
[b] SEM = Standard Error of the Mean with 382 and 94 degrees of freedom, respectively.

TABLE 6.

Effect of pH of poultry bait: ocean evaluation[a].

pH	Male Crabs	Female Crabs	Total Crabs
6	1.479	2.500	3.979[1]
7	1.312	2.562	3.875[1]
8	1.667	1.729	2.896[2]
9	1.667	2.521	3.687[1,2]
Statistics (pooled)			
pH Effects (P values)	.8359	.3883	.045
SEM[b]	.278	.398	.297

[1–2] Means within columns with no common superscript differ significantly ($P < .05$).
[a] Average number of blue crabs harvested/pot/day.
[b] SEM = Standard Error of the Mean with 181 degrees of freedom.

TABLE 7.

Effect of betaine: ocean evaluation[a].

Factor	Male Crabs	Female Crabs	Total Crabs
Betaine	1.198	1.989	3.187[2]
No betaine	1.364	2.667	4.031[1]
Statistics (pooled)			
Betaine effect (P values)	.550	.090	.005
SEM[b]	.197	.281	.210

[1 2] Means within columns with no common superscript differ significantly ($P < .05$).
[a] Average number of blue crabs harvested/pot/day.
[b] SEM = Standard Error of the Mean with 181 degrees of freedom.

TABLE 8.

Comparison of protocol's ability to detect significant treatment differences: attraction tray trial 2 and ocean evaluation.

Factor	Attraction Tray Trial 2	Ocean Evaluation
	P Values	
Sex	.7735	.001*
pH	.1995	.045*
Meat	.1525	.381
Betaine	.8449	.005*

* Indicates significant differences detected in the parameter.

TABLE 9.

Effect of baits on the organoleptic indices of crabmeat[a,b].

Bait Treatment	Perceived Mean Difference from Control[c]			Perceived Mean Difference from Blind Control[c,d]		
	Aroma	Texture	Flavor	Aroma	Texture	Flavor
Menhaden (blind control)	2.387	2.710	2.677	.000	.000	.000
Fresh poultry	2.548	2.226	2.129	.161	−.484	−.548
Fermented poultry	2.226	2.387	2.645	−.161	−.323	−.032
		Statistics (pooled)				
Bait treatment effect (*P* value)	.630	.423	.242	.689	.357	.222
SEM[e]	.237	.264	.256	.264	.241	.249
Minimum Significant Difference from Control Required for Dunnett's T	.753	.841	.815	.839	.767	.791

[a] Lump and backfin crabmeat obtained from crabs consuming various baits.
[b] Mean of 31 consumer panelists.
[c] Rankings of perceived differences based on a seven-point scale.
[d] Blind control presented as sample.
[e] SEM = Standard Error of the Mean with 90 degrees of freedom.

crabs will choose a poultry bait product between 40% and 67% of the time, depending on the bait formulation. Poultry mortality or poultry mortality silage can be used directly as bait or can be used as a base component to which a variety of flavor enhancements, aquatic products, or aquatic waste products could be added to increase the overall attractant quality of the material. Binding agents utilized and poultry meat formulation (fresh versus fermented) had no effect in any trial on the ability of the bait products to attract blue crabs. Preferences for bait pH, preference differences between male and female crabs, as well as a reduced preference for supplemental betaine were documented in one or more trials. No off flavors in the crabmeat are associated with the use of the alternative poultry baits. Bait durability of at least 5 days in the aquatic environment has been achieved with current binding systems. This level of product durability allows realistic ocean testing of various alternative poultry bait formations *versus* traditional bait products to proceed. In addition, the potential for use of poultry based products as baits for other aquatic species is possible and requires further investigation.

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THE FIRST LARGE-SCALE FISHERY-INDEPENDENT SURVEY OF THE SAUCER SCALLOP, *AMUSIUM JAPONICUM BALLOTI* IN QUEENSLAND, AUSTRALIA

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ABSTRACT The saucer scallop, *Amusium japonicum balloti*, is a valuable component of a multispecies trawl fishery off the Queensland east coast. In recent years, a decline in catch rates resulted in the closure of small areas within the fishing grounds (preservation zones) and the allocation of funding for a large-scale fishery-independent survey. The first survey based on a stratified random survey design is reported in this paper. The survey found relatively low densities within the strata as compared with similar species in other parts of Australia and elsewhere in the world. The position of two of the three preservation zones was extremely appropriate, because together they contained 20% of the over-all numbers caught in the whole survey. The highest densities in the remaining fishing ground occurred in the inshore north and central strata. The southern sites were characterized by a high proportion of very low or zero density sites. If a previously published gear efficiency parameter on this species and gear is used, then absolute adult abundance values are within the same order of magnitude as the commercial catch, and fishing pressure may be high. The application of this measure of gear efficiency to calculate absolute adult abundance estimates is discussed. Four methods of estimating confidence intervals are discussed. The survey was extremely successful in terms of coverage of the major scallop grounds and production of density estimates with low coefficients of variation.

KEY WORDS: scallop densities, stratified random, fishery-independent, bootstrap confidence intervals

INTRODUCTION

The fishery for saucer scallops, *Amusium japonicum balloti*, is an important component of a multispecies trawl fishery on the east coast of Queensland. Annual landings average about 1,200 tons of adductor muscle meat, with a landed value in excess of $25 m (Williams 1997). The scallop fishery takes place mainly between 21°S and 27°S, in depths ranging from 20 to 60 m (Fig. 1). It is regulated through input controls, which include entry limitation (which applies to the entire Queensland east coast trawl fishery) and minimum legal size limits designed to optimize yield per recruit (Dredge 1990, Dredge 1994). The fishery was characterized by 24-h fishing operations until 1988, but was limited to night-time only operations thereafter. Three 10 by 10-minute areas were closed to trawling to act as broodstock reserves in 1989, but were repealed 15 months later because of policing difficulties. Similar closures were again introduced in 1997 as a response to serious declines in catch rates and were still in place at the end of 1999.

Saucer scallops have been shown to spawn in winter and spring, coinciding with water temperature changes. It is probable that saucer scallops are serial spawners, with females spawning more than once in a season (Dredge 1981). Growth is rapid, with most animals attaining sexual maturity at a shell height of 90 mm or toward the end of their first year of life (Williams and Dredge 1981, Dredge 1981). Natural mortality rates of adults are high, with an instantaneous rate between 0.020 and 0.025 $week^{-1}$ (Dredge 1985a), suggesting that few saucer scallops survive more than 3 years (Heald and Caputi 1981). It is assumed that the bulk of each year's catch and spawning population comes from a single year-class which is fished at late 0+ and 1+ animals.

Queensland's saucer scallop stock was first fished in the mid-1950s, when prawn trawlers working out of Hervey Bay took appreciable quantities (Ruello 1975). Although annual landings have not shown the spectacular variation often associated with scallop fisheries (Hancock 1979), catch rates declined by an order of magnitude in the period 1980–1988 (Dredge 1994) and declined further in the mid-1990s (Fig. 2 and Williams 1997). The fishery is seasonal. Maximum catches and catch rates occur in early summer months, when young of year (YOY) animals first recruit into the fishery, and adductor meat condition is at its peak (Williams and Dredge 1981). Variable minimum legal size limits apply to the fishery, with size limits being reduced from 95 to 90 mm shell height in November each year, for a 6-month period to maximize yield per recruit. This has the effect of amplifying the early summer effort pulse (Dredge 1994).

Average catch rates observed in late 1996 and early 1997 were less than half of the 1988–1995 average for that time of year (Fig. 2). This decrease in catch rates was of sufficient concern to managers and fishers to generate support for a gazettal of emergency broodstock closures. Resources were then allocated for a large-scale survey designed to collect data on scallop densities, size composition, distribution, and estimated abundance of saucer scallops in the main fishing grounds. The data were to be collected to establish baseline information on the state of the saucer scallop resource.

Many bottom trawl surveys conducted to estimate fish stock sizes use a stratified random design with stratum boundaries defined by depth-ranges, species-specific distribution, or management areas. Confidence intervals for stock size estimates made from data collected in such surveys are usually estimated on the basis of sample error being normally distributed, which has been shown to be the limiting distribution for the stratified mean (and total) when the central limit theorem is applied to sampling a finite

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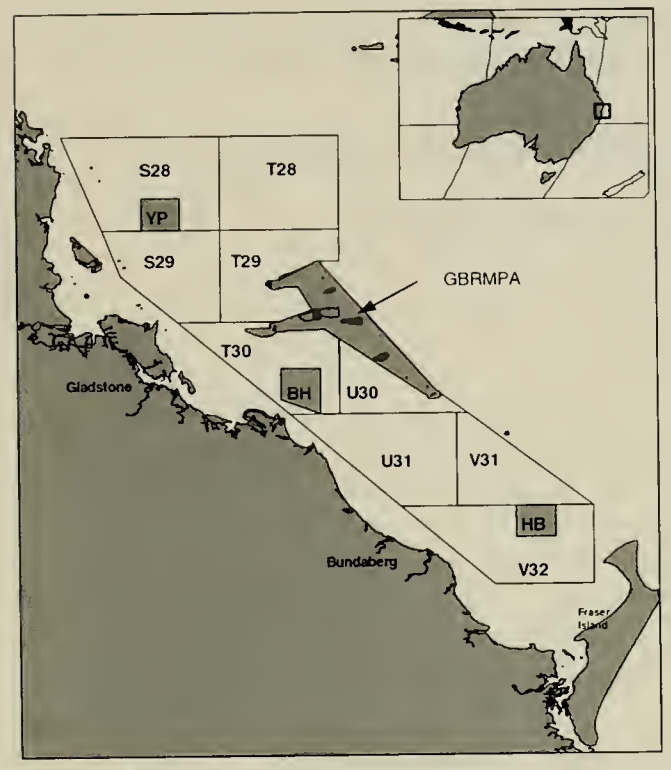

Figure 1. Map of saucer scallop survey area as well as nearest local towns and strata areas and codes within the survey. The arrow indicates the Capricorn–Bunker reserve area of the Great Barrier Reef Marine Park Authority (GBRMPA) that was not included in the survey. Shaded strata (YP, BH, and HB) are the scallop preservation zones. Inset shows survey position within Australia. T28 is 30 × 30 min grid.

population (Cochran 1977). Sampling strategies that involve relatively small sample numbers per stratum when sample catches have skewed frequency distributions may result in biased confidence intervals. Several authors have suggested modeling the distribution of estimates from surveys using bootstrap resampling methods (e.g., Effron 1982). Bootstrap confidence intervals do not require a distributional assumption for their construction and, thus, can be used to evaluate the standard normal distribution theory intervals. In this paper, estimates of saucer scallop stock densities and associated confidence limits have been analyzed using the classic Cochran approach, the bootstrap-t method (Effron 1982) and two skewness adjusted methods suggested by Hall (1992).

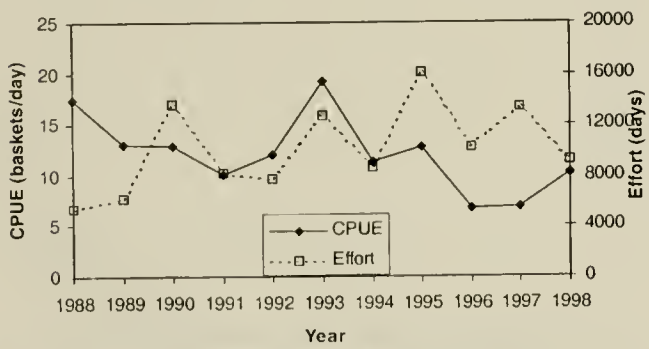

Figure 2. Commercial saucer scallop catch rate (baskets per day) and effort (day) statistics for the survey area from 1988 to 1998.

METHODS

A survey of adult and juvenile saucer scallop abundance was undertaken in the period from 5 to 16 October 1997 using four chartered commercial scallop trawlers. A total of 6,700 n. miles^2 (23,000 km^2) offshore from north of Yeppoon to southern Hervey Bay was surveyed (Fig. 1, Table 1). The Great Barrier Reef Marine Park Authority (GBRMPA) Management Area B in the Capricorn Bunker area (a conservation zone covering some 500 n. miles^2 or 1,800 km^2) on the eastern edge of the survey area was not surveyed. The dates of the survey were chosen so that the survey was centered around neap tides (beginning just after the new moon and ending before full moon) to minimize the effects of tide on scallop catchability. October was chosen to optimize 0+ recruit catch before the main fishing season, when the size limit reduces from 95 to 90 mm shell height, and to minimize the probability of adverse weather affecting the survey. The area covered by this survey has, on average, corresponded to more than 90% of the Queensland landings in the past 10 years. Trawls were of constant duration (20 min) at constant speed, and, as best could be arranged, in a straight line. Distance covered and exact locations were recorded using onboard Global Positioning Systems (GPS).

Design

This is the first large-scale scallop survey of its type conducted in Queensland waters (Fig. 1). As a result, no estimates of density variance over the whole area were available. However, two separate smaller (unreported) surveys had previously been undertaken. Sampling in one, which took place in 1989, was not sufficiently randomized to provide estimates of variance in saucer scallop abundance in the survey area. A better designed survey of the two preservation areas within the survey area off Bustard Head (BH) and Hervey Bay (HB) (Fig. 1) was completed in early 1997. Further historical information on catches and catch rate variation from the fishery was available in terms of commercial saucer scallop catch (baskets) and effort (number of days fished) from commercial logbooks. These data can be summarized spatially in 30 × 30-min grids.

The survey area was divided into 12 strata loosely based on the 30′ × 30′ grids. The three 1997 broodstock preservation zones (Fig. 1), labeled HB, BH, and YP, were treated as separate strata. Sample intensity within the strata was based upon a weighting process, using a range of commerical catch rates (catch per unit effort, CPUE) multiplied by stratum area (Table 1). Various forms of CPUE were considered. They included:

1. mean annual CPUE from 1988–1996;
2. mean CPUE between October to December from 1988–1996;
3. variance of mean annual CPUE from 1988–1996; and
4. variance of mean CPUE between October to December from 1988–1996.

In all cases, the relative weights were extremely similar and method 1 was chosen. The final weights (Table 1) were modified to ensure that no stratum weight allowed for less than 2% of the total sampling effort.

The total number of sites to be sampled was limited by financial resources, which allowed four boats to be chartered for 12 days. It was estimated that an average of 10 sites per boat per night could be sampled. This translated to a total of 480 sites that could be sampled, of which 45 would be set aside for a preliminary

TABLE 1.

Description of strata, sampling intensity, and the survey results in terms of mean densities and stratum variance for 0+ and 1+ age group saucer scallops.

Stratum Identifier	Area (1,000 m^2)	Weight	Number of Sites Sampled	Mean Relative Densities (Number · m^{-2})		Stratum Variance	
				0	1+	0	1+
S28	3284747	0.17	65	0.0073	0.0057	0.000279	0.000068
T28	3092947	0.17	46	0.00259	0.0036	0.000012	0.000123
S29	2349482	0.10	44	0.0022	0.0011	0.000004	0.000002
T29	2362447	0.07	29	0.00271	0.0026	0.000013	0.000006
T30	2079926	0.10	37	0.0053	0.0057	0.000031	0.000029
U30	1292695	0.05	19	0.00356	0.0036	0.000017	0.000040
U31	293695	0.12	52	0.0017	0.0030	0.000017	0.000022
V31	1938751	0.07	26	0.00138	0.0003	0.000020	0.000001
V32	2881528	0.10	45	0.0016	0.0012	0.000006	0.000002
YP	347998	0.02	11	0.0018	0.0027	0.000001	0.000006
BH	441462	0.02	13	0.0079	0.0260	0.000030	0.000486
HB	347998	0.02	10	0.0053	0.0100	0.000062	0.000174
Total	23356933	1.00	397				

calibration experiment and subsequent calibrations. The final number of sites completed in each stratum is given in Table 1.

Previous work has shown that saucer scallops occur in beds with a maximum density of about one per m^2. Beds are separated by areas of zero or extremely low densities (Dredge 1988). The only known saucer scallop beds that have been mapped in detail have ovoid spatial distributions, with a width across the beds of up to 4 km (Dredge 1985b). Given this information, sites were selected on the basis of being 4 km (2 nm) apart or more. Subject to this rule, the sample sites were randomly chosen within each stratum. Because very little detailed knowledge of the grounds was available to research staff, several backup sites were randomly chosen to replace sites that fell on untrawlable grounds.

Description of Boats and Gear

The four commercial trawlers and their skippers had extensive histories of involvement in the Queensland scallop fishery. Table 2 summarizes basic specifications of the vessels and the gear they used. Small mesh trawls (ca. 50-mm stretched mesh) were used for all sampling to capture animals much smaller than the commercial scallop fleet normally takes.

Calibration Experiment

Because four vessels using different gear configurations were to be involved in the survey, it was necessary to calibrate their relative fishing power. It was originally intended that the vessels undertake 10 side-by-side trawls on the first night of the survey. The calibration experiment was actually completed on the survey's second day, during daylight hours, as a consequence of poor weather conditions experienced on the first night. The calibration work took place over a bed of scallops in the general area of 151°38.50′E and 23°27.70′S. Calibration involved having boats trawl side by side, undertaking 20-min shots, and having all scallops counted and measured at the completion of each shot. Start and end fishing points for each trawl were recorded from GPS. The relative (port–starboard) position of each boat was randomly determined for each trawl. Because the variability between the boats was not known, it was not possible to calculate the number of shots needed for a statistical expression of the difference in fishing power between vessels.

To investigate the possibility that the vessels' power changed during the survey, it was anticipated that some form of recalibration would occur during or after the survey. In practice, this proved impossible, because the vessels were widely separated throughout most of the survey.

TABLE 2.

Survey vessel characteristics.

Vessel Number	Length	Rated Main Engine Power (hp)	Trawl Gear Used in Survey
1	18.10 m	350	2 by 14-m and 1 by 18-m head rope length
2	15.84 m	350	4 by 10-m head rope length
3	15.66 m	300	5 by 8-m head rope length
4	15.24 m	300	2 by 9-m, 2 by 7-m head rope length

Data Collection

The survey proper was conducted by having each vessel working in an area that approximated about a quarter of the over-all survey area. Survey vessels steamed to each survey site sequentially, and the skipper determined if it could be trawled. If the ground was workable, a 20-min trawl shot was undertaken into the prevailing tidal flow when possible, at fixed speed. The starting and finishing positions were recorded using GPS (accuracy ± 60 m). The number of saucer scallops taken in all nets was counted at the conclusion of the trawls. Scallops from either one or all nets (depending on sample size) were measured to the nearest mm. Site characteristics were identified on the basis of: unique shot number, site number, date, time, starting trawl latitude and longitude from GPS, end trawl latitude and longitude, distance covered, bearing, depth, and trawl duration. All sampling was done from sunset to sunrise between the hours of 18h00 and 07h00.

Analysis

A generalized linear model was undertaken of the natural log of the catch rates from the calibration experiment incorporating, as factors, the different vessel, sample sites, and their position relative to each other using the PROC GLM module of SAS (SAS 1991). A power test of the resultant ANOVA was undertaken to test what sample size would be needed in the future to detect a possible difference between vessels (Thomas unpubl.).

The swept area for each trawl was calculated from the distance trawled and the swept width estimate for each vessel, assuming gear spread of 60–70% of the full headrope length for the four vessels, and using the experience and observations of individual vessel skippers to make estimates for each boat. Relative densities (numbers · m^{-2}) at each site were estimated using numbers caught and the area swept.

The size-frequency plots for scallops from each 10-min by 10-min block within the region and for the over-all pooled data (Fig. 3) are clearly bimodal. The modes and size distributions are consistent with known growth rates of scallops (e.g., Williams and Dredge 1981), with the first mode being in the size range expected of young of bear saucer scallops (i.e., scallops spawned in the winter immediately preceding the survey) and the second mode representing 1+ and older saucer scallops. For analysis purposes, animals smaller than 78 mm were assumed to be less than 1-year old (0+ year class) and those greater and equal to 78 mm more than 1-year old (1+ and older year classes, hereafter referred to as "1+" only). The bimodal size frequency distribution was consistent throughout the region.

Survey data used to estimate relative population densities were analyzed initially according to Cochran (1977). The initial design stratum weights (w_h) were used in the analysis. The stratified mean and its standard error is therefore given by:

$$\overline{X}_{st} = \sum_{h=1}^{12} W_h \overline{X}_h,$$

and

$$se(\overline{X}_{st}) = \sqrt{\sum_{h=1}^{12} W_h^2 S_h^2 / n_h},$$

where

$\overline{X}_{st}$ is the stratified mean (numbers · m^{-2});

$se(\overline{X}_{st})$ is the Studentized version of the estimated standard error (Cochran 1977),

$W_h = n_h/N$ is the weight for stratum h with n_h the number of stratum sites

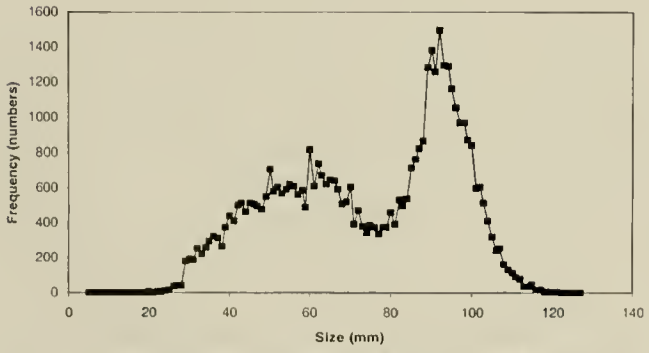

Figure 3. Size-frequency plot of all animals measured within the survey.

planned to be sampled out of a total of N sites; and

S_h^2 is the stratum variance.

Ninety-five percent confidence intervals were calculated using four different techniques:

1. assuming the population abundance has a near normal distribution within each stratum. An approximate 95% confidence interval (i.e., $\alpha = 0.05$) for the population mean μ is, therefore, given by:

$$\overline{X}_{st} \pm t(df,\alpha) se(\overline{X}_{st})$$

with *df* being the appropriate degrees of freedom for the t-distribution (Cochran 1977)

2. a general alternative of the above method through the use of bootstrapping procedures. This was conducted by resampling the Studentized version of the stratified mean; that is, use the bootstrap-t method of calculating confidence intervals (Effron 1981). Ten thousand bootstraps were used in this process.
3. an alternative to these two methods of Studentized confidence intervals is a skewness corrected cubic transformation of the bootstrap-t method (Hall 1992), and
4. bootstrapping, again with 10,000 bootstraps, the above skewness corrected bootstrap-t method (Hall 1992). The latter two methods have been shown to be less biased than the first two methods through simulation modeling.

These methods of calculating confidence intervals are well discussed and explained in such texts as Manly (1997) and Fletcher and Webster (1996).

RESULTS

Calibration Experiment

The full general linear model with vessel, relative position, and trawl site explained 51% of the variance between the vessel's catch rates. However, only the factor "sample site" was significant ($P <= 0.05$) and explained more than 45% of the variance. Vessel as a factor was not significant, because there was very little difference in catch rates between vessels, and the "between vessel" variance was extremely small. *A posteri* power tests suggest that 77 calibration trawls would have been needed to detect a possible difference between vessels at a power of 0.6. The residuals of the models were normally distributed.

Survey Analysis

Samples were taken from 397 of the planned 480 sites. Sites were not sampled either through unsuitable bottom conditions or restrictions on vessel time. The shortfall in sampling did not skew the planned sampling frequency between strata. Scallop densities for the total, commercial, and preservation areas are given in Tables 3 and 4. The total area has been defined as the sum of the preservation and commercially accessible areas. Average trawl speed was 2.38 ± 0.38 knots.

The frequency distribution of densities per stratum for both 0+ and 1+ year olds are given in Figures 4 and 5, respectively. There is some suggestion of a change in frequency distribution of densities from north to south. The northern sites tended to have a greater range of densities including some high values; whereas, the southern sites, especially V31, had many sites with few or no saucer scallop. The preservation zone, BH, also had a large proportion of high density sites for both 0+ and 1+ year olds.

TABLE 3.

Relative densities of 0+ year old saucer scallop for the total survey area, the commercial areas only and the preservation areas alone.

	Total Area	Commercial Areas	Preservation Area
Mean density (numbers · m^{-2})	0.0035	0.0034	0.0050
Standard error	0.0004	0.0004	0.0009
Coefficient of variation	11.4%	12.2%	19.5%
Lower and upper 95% confidence limit: NT	0.00275:0.00435	0.00250:0.0040	0.00305:0.00685
Upper and upper 95% confidence limit: BT	0.00295:0.00475	0.00265:0.0045	0.00340:0.00760
Lower and upper 95% confidence limit: NH	0.00290:0.00485	0.00260:0.0046	0.00330:0.00780
Lower and upper 95% confidence limit: BH	0.00295:0.00505	0.00265:0.0077	0.00345:0.00945

Confidence limits were calculated using four methods: NT analysis using Cochran's (1977) t-distribution method; BT, the bootstrap-t method; NH, the skewness corrected cubic transformation of the bootstrap-t method; BH, the bootstrap version of the skewness corrected bootstrap-t method.

Densities per site for both 0+ and 1+ year olds are shown in Figures 6 and 7 respectively. To provide contrast in the data, the classification scales are not linear. Most of the densities are very low with a few patches of much higher values. In some cases, these large densities coincide with the preservation areas.

DISCUSSION

Production levels of saucer scallop from Queensland waters have been relatively stable compared with most natural fisheries for scallops (see reviews in Shumway 1991), although catch rate have varied considerably (Dredge 1994). The dramatic decline of catch rates in 1996 triggered a series of responses. The two significant decisions made were the creation of three preservation zones and financial support for a large-scale independent survey. The survey was designed to develop baseline data on scallop populations on the main saucer scallop commercial fishing ground and is the first of its kind undertaken in Queensland waters. Its main aim, to produce a relative recruitment index, requires that these surveys be continued over time and that estimates of abundance are obtained with narrow confidence intervals. The survey covered an area from which more than 90% of Queensland saucer scallop landings are, on average, taken. Estimates of population size are, therefore, highly relevant to the management of the fishery.

The survey was undertaken in October to optimize the size of recruits (and, therefore, their catch) before the main fishing season, when the size limit changes from 95 to 90 mm. It was designed to give data on relative density and relative abundance.

The preservation zones together contained 20% of the estimated animals in the survey. Most of these were within the central (BH) and southern (HB) preservation zones, with the highest density of 0+ and 1+ year olds being recorded in BH. However, the northern zone (YP) contained few scallops. Subsequent to this survey and as a consequence of information obtained from the survey, the YP preservation area was moved to a higher density area within stratum S28. Overall, therefore, if the animals within these zones do seed surrounding areas and illegal catches are minimized, they are well situated to offer an effective mechanism for protection of broodstock and reduce the risks of recruitment overfishing.

There is a widespread, but little published belief that recruitment of scallops is of such irregularity and unpredictability that there is little purpose in attempting to manage broodstock levels to maintain recruitment. Orensanz et al. (1991) discussed scallop stocks in the context of four recruitment categories—steady stocks, cyclical stocks, irregular stocks, and spasmodic stocks, and stated that most scallop stocks belong in the latter group, with irregular pulses of high abundance followed by periods of scarcity or collapse. There are, however, examples of scallops recruitment levels being related to parent stock levels. McGarvey et al. (1993) described a stock–recruitment relationship for *Placopecten magellanicus* in the Georges Bank area, and there is clear graphical evidence of increased recruitment with increased parental population size in *Platinopecten yessoensis* (Ito and Byakuno 1990). Mace and Sissenwine (1993) refer to Atlantic stocks of *Placopecten magellanicus* as requiring relatively low spawner per recruit levels to minimize the risk of overfishing.

The introduction of broodstock preservation areas when catch rates of saucer scallops were depressed well below those historically observed were consistent with a belief that recruitment overfishing was a possibility in this species. Results from this survey show that about 20% of the population are protected from fishing.

However, little is known of the dynamics of larval transport and

TABLE 4.

Relative densities of the 1+ and older saucer scallop for the total survey area, the commercial areas only and the preservation areas alone.

	Total Area	Commercial Areas	Preservation Areas
Mean density (numbers · m^{-2})	0.0038	0.0033	0.0129
Standard error	0.0004	0.0087	0.0025
Coefficient of variation	9.9%	11.2%	19.2%
Lower and upper 95% confidence limit: NT	0.00315:0.00465	0.00245:0.00385	0.00795:0.01760
Upper and upper 95% confidence limit: BT	0.00330:0.00500	0.00260:0.00435	0.00860:0.01860
Lower and upper 95% confidence limit: NH	0.00300:0.00495	0.00255:0.00420	0.00840:0.01975
Lower and upper 95% confidence limit: BH	0.00325:0.00555	0.00260:0.00685	0.00875:0.01915

Confidence interval codes as in Table 3.

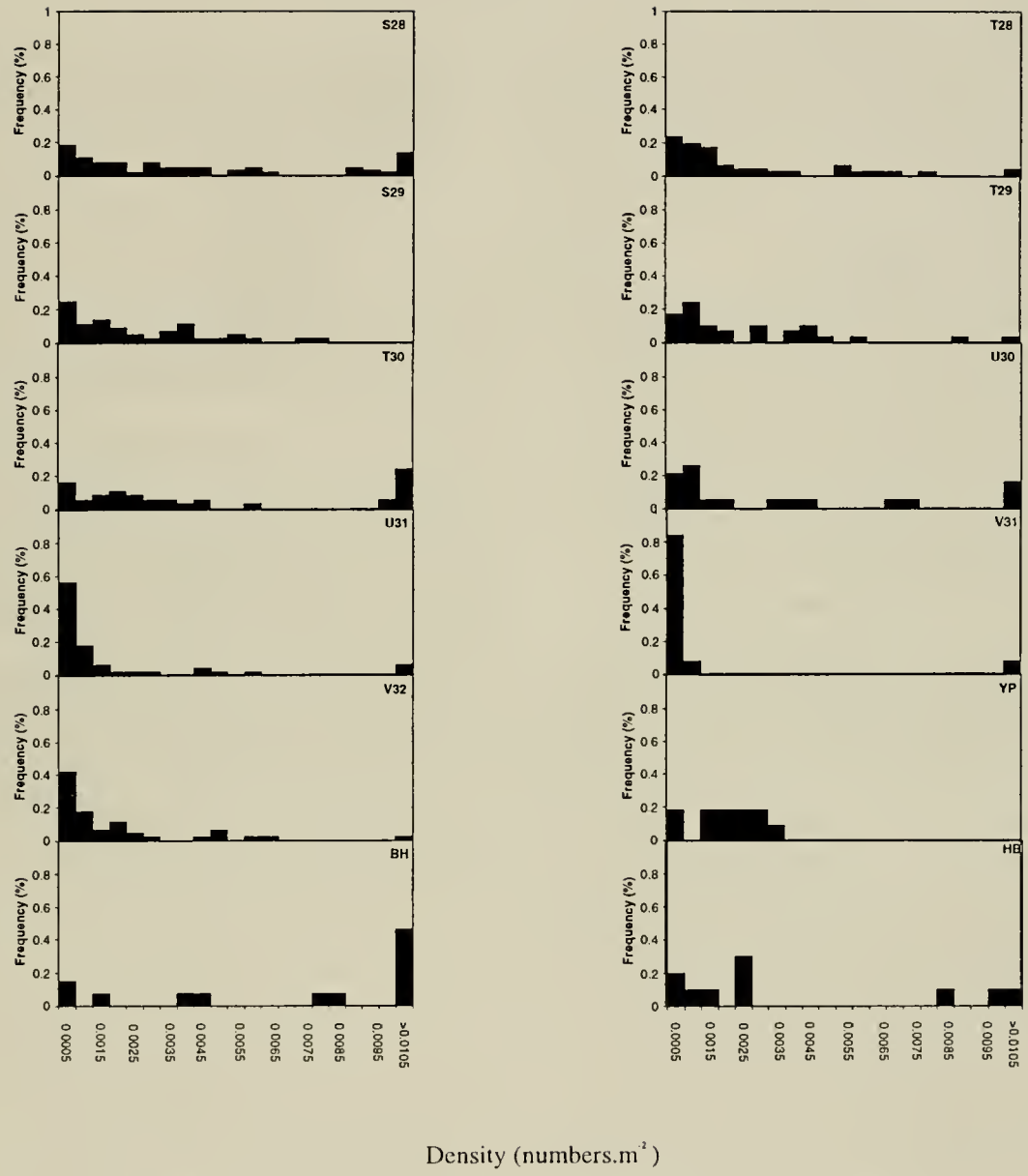

Figure 4. Density-frequency plots of catch (numbers · m^{-2}) of 0+ year olds per stratum.

recruitment within Queensland waters and so the long-term value of the preservation zones is hard to estimate. The abundance pattern in Shark Bay, Western Australia of 1990 had a core of very high abundance surrounded by an area of relatively high abundance, suggesting that larvae were contained within a well-defined eddy at settlement (Joll 1994). Dredge (1988) suggested that a gyre in Hervey Bay, which falls in the survey area described in this paper, might act to trap larvae. Caddy (1979) hypothesized that recruitment to the Bay of Fundy fishery was positively influenced by the degree of retention of larvae within a gyre. Further study into the oceanography of the survey region would be required to understand the dynamics of larval transport fully, settlement and recruitment.

Within the main fishing grounds, the highest relative density strata within the survey were the inshore north and central areas. Apart from a few high relative density sites, the southern strata contained very few saucer scallops. This over-all low relative density within a stratum is most notable in V31 where more than 80% of the sites had densities of less than 0.001 saucer scallops per m^2 of 0+ and 1+ year olds. This contrasted with the two highest relative density sites for juvenile and adults (S28 and T30) in which more than 10 and 20%, respectively, of the sites contained relative densities higher than 0.01 scallop per m^2 of 0+ and 1+ year olds, respectively.

The availability of gear efficiency estimates for capture of saucer scallops from another study (Joll and Penn 1990), makes it possible to estimate absolute abundance values of legal size animals, assuming 100% selectivity for animals >90 mm and no swept area changes during the survey. The relative density from the survey of animals >90 mm in the commercial areas were 0.0010 scallops · m^{-2}. Given the range of values estimated in Joll and Penn (1990), efficiency values of 0.5 and 0.6 were tested and give legal size animal abundances in the commercial areas of 0.0020 and 0.0017 scallops · m^{-2}, respectively.

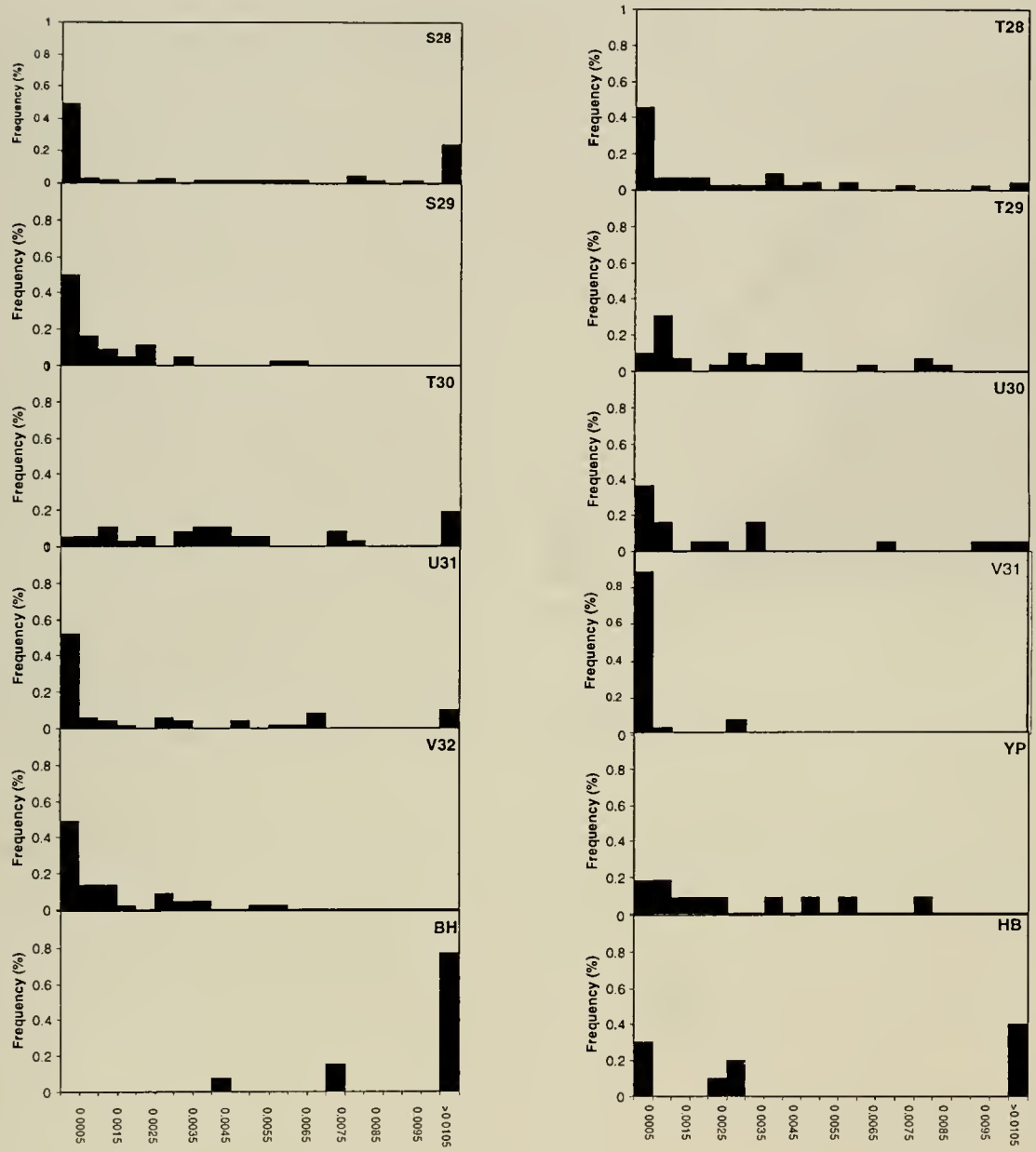

Figure 5. Density-frequency plots of catch (numbers · m^{-2}) of 1+ year olds per stratum.

Because the survey estimates relative densities, it is difficult to compare results with other studies. Minchin and Mathers (1982) found densities of up to eight *Pecten maximus* scallops per m^2 in Ireland. In the same region, commercial fishable concentrations were considered to be around 0.1 to 0.2 scallops per m^{-2} (Gruffydd 1972), although improvements in fishing gear efficiency may have lowered this threshold slightly. Buestal et al. (1985) found average densities from dredge samples to be about 0.6 scallops · m^{-2} in the Bay of St. Brieuc. Within-bed densities have been well published with a summary of some of these in Brand (1991) and Oresanz et al. (1991). Most of the densities of those scallop species recorded in these texts are a few orders of magnitude higher than we observed and were directed at known high-density beds. Surveys in the 1980s in Port Philip Bay, Australia also produced estimates of densities ranging from 0.01 to 0.6 scallops · m^{-2} (e.g., Gwyther and McShane 1985). These variations in abundance may reflect intrinsic differences in behavior and density tolerances between species of *Amusium* and less mobile pectinids. Joll (1994), however, reported estimated *Amusium* densities of scallops in the area of highest abundance in the 1990 and 1991 surveys as 6.7 scallops m^{-2} and general densities at a time of very high stock abundance of 0.57 1+ scallops m^{-2} and 1.42 0+ scallops m^{-2}. Joll and Penn (1990) reported densities of scallops of 0.08–0.09 m^{-2} in an area of Shark Bay in 1986. These studies were on beds of normal

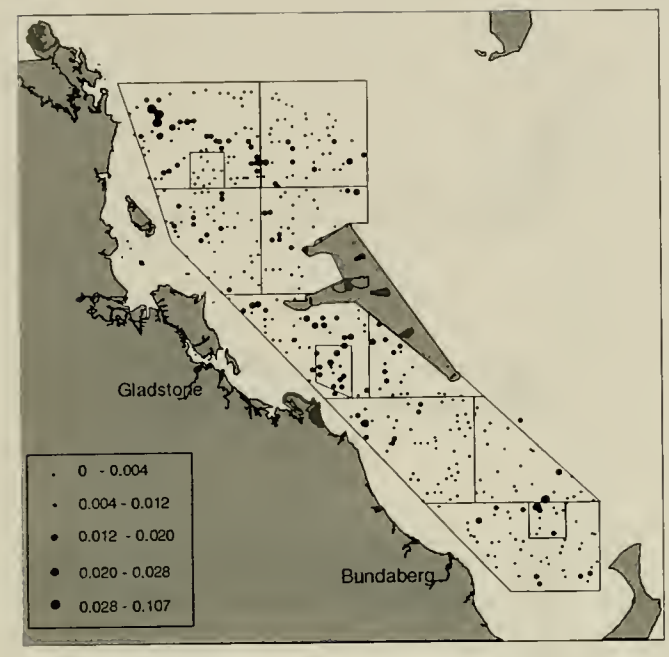

Figure 6. Densities (numbers · m^{-2}) of the 0+ year old saucer scallops caught in the survey.

abundance within the area at the time. In Wilson and Brand (1995), the over-all preseason density of commercial size *Pecten maximus* was around three scallops · 100 m^{-2} based on survey areas covering six fishing grounds.

Generally, variances of mean densities of 0+ and 1+ scallops within strata were low. This suggests that stratum borders were well situated. However, this low variance was also influenced by the over-all low densities. The over-all coefficient of variation of 11.4 and 9.9% of 0+ and 1+ year old densities was also very low, suggesting relatively uniform (but low) densities. Confidence intervals of estimates are consequently fairly small, and there was little difference between the different methods of estimating intervals. An exception is the upper limit from the bootstrapped version of Hall's cubic transformation method. Simulation tests of the data would be the only method of explaining this difference, but were not done in this study. In other studies, simulation of groundfish trawl surveys of Georges Bank and the Scotian Shelf data indicate that the bias-corrected and accelerated confidence limits may overcorrect for the trawl survey data and that the percentile limits were closer to expected values (Smith 1997). These methods were therefore not applied to these data.

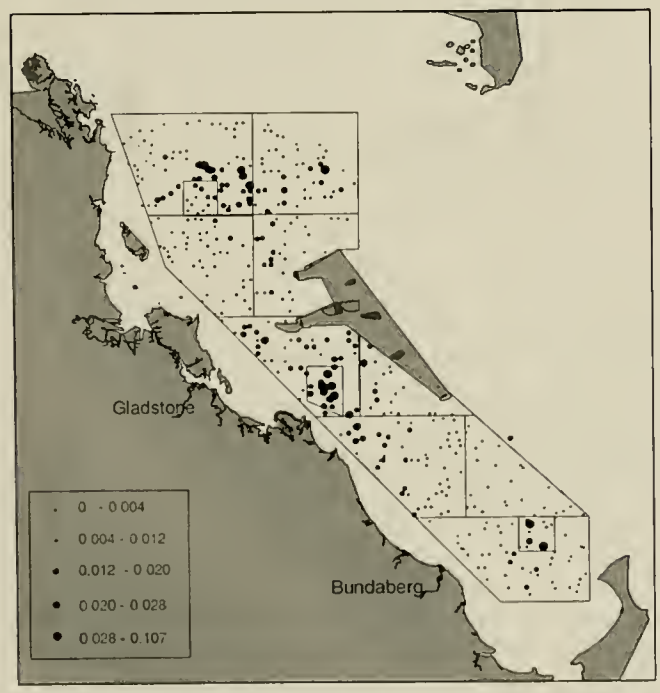

Figure 7. Densities (numbers · m^{-2}) of the 1+ and older year old saucer scallops caught in the survey.

Several biases and sources of variance need to be assessed for future surveys. Most importantly is the swept width of each vessel's trawl gear, which is based on sparse information. Large biases can result, and uncertainties may be underestimated if scientists treat catchability coefficients as constants without error and subsequently use survey biomass values as absolute estimates of biomass (McAllister and Pikitch 1997). Because the gear efficiency parameter used in this study was based on Leslie and DeLury methods (Leslie 1952, Delury 1947), their assumptions should be noted. These are that the target species has constant catchability over the sampling period, the fishing effort is distributed uniformly over the fishing ground, the fishing methods do not change, the target population is closed, and the landings and effort are reported correctly. Biases in estimates of fishable biomass cannot be corrected without knowing the cause (Miller and Mohn 1993).

A further source of bias is mesh selectivity. Experiments made in Shark Bay on the relative selectivity of prawn and scallop mesh was undertaken in November 1985 (Joll 1987). Prawn mesh of 50 mm and scallop mesh of 100 mm (stretch mesh) were used. The length frequency data show that prawn nets catch scallops as small as 30 mm, but efficiency of capture for such small scallops is unknown. Scallop and prawn mesh have a similar selective efficiency for scallops of 90 mm or greater. The scallop mesh selectivity is negligible below 70 mm or less (Joll 1987). This means that juveniles below a certain size were not being caught by the gear used in this survey and that there is a selectivity ogive of unknown slope and configuration above this size. It may, therefore, be difficult to determine whether a change in estimated survey density between years is attributable to a change in true numbers or a change in recruitment timing. Further work on selectivity and recruitment timing is, therefore, justified.

The survey reported in this paper offers some insight into distribution, recruitment, and density variation of saucer scallops throughout their major fishing ground. The results, however, will be of real value as the survey is repeated over time, and a history of fishery-independent recruitment processes, with their linkage to climate and other variables, is developed.

ACKNOWLEDGMENTS

This survey was funded by the Queensland Department of Primary Industries. The authors thank all those who went on the survey and collected the data, often working long hours. Also, the skippers and their crews for their cooperation and helpfulness, and Dr. David Mayer for his useful comments on the design and subsequent analysis.

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SEASONAL VARIATIONS IN CHEMICAL COMPOSITION OF THE FEMALE GONAD AND STORAGE ORGANS IN *PECTEN MAXIMUS* (L.) SUGGESTING THAT SOMATIC AND REPRODUCTIVE GROWTH ARE SEPARATED IN TIME

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ABSTRACT Glycogen, protein, and lipid composition were determined in the female part of the gonad, striated adductor muscle, and digestive gland during 1 year in great scallops (*Pecten maximus*). The scallops were sampled from Raunefjorden outside Bergen on the west coast of Norway. From the spring phytoplankton bloom in March to June, gonad growth, spawnings, and rebuilding took place; whereas, no somatic growth was seen during this period. A marked increase in digestive gland protein during the spring bloom may have represented an increase in digestive capacity induced by the increased food levels. Between June and August, a concentrated period of somatic growth and increased storage was seen; whereas, the female gonads showed no signs of rebuilding, because they decreased due to spawning activity. Gonad rebuilding took place between October and December. Reproduction in many populations of *P. maximus* is regarded as more or less continuous, since individuals with filled gonads can be found all year. However, for the population in this study, it seemed that no energy was allocated to the gonads in the period starting in June, with somatic growth and storage, until the gonad rebuilding in October, even though the gonads were still containing presumably growing oocytes. From these results, we suggest that switches in energy allocation divide the season in two parts in these scallops: (1) priority of reproductive growth from October to June; and (2) priority of somatic growth and storage from June to October.

KEY WORDS: adductor muscle, chemical composition, digestive gland, energy storage, female gonad, growth, *Pecten maximus*, reproduction

INTRODUCTION

Scallops, as do other marine bivalves, exhibit cyclic changes in both reproductive and somatic body components as a consequence of the seasonality of environmental conditions in temperate areas (Barber and Blake 1991). Reproductive cycles are based on the build up of gametes and release at a time with favorable conditions for larval growth and survival. The seasonality in temperature and food conditions results in annual cycles in build up of somatic and storage material that is later utilized during periods of food shortage or also to support gametogenesis. In scallops, it has been demonstrated that energy is stored in the adductor muscle and digestive gland, since weights of these tissues increase during periods with excess food and decrease during periods with strong gametogenesis or low food levels (Ansell 1974, Barber and Blake 1981, Comely 1974, Pazos et al. 1997, Robinson et al. 1981, Taylor and Venn 1979). Chemical analyses of the storage organs have revealed that glycogen and protein in the adductor muscle and lipid in the digestive gland are the major energy reserves (Barber and Blake 1991).

Timing of reproduction and the relationship between gonad growth and variations in the storage organs have been studied in several scallop species. Generally the storage organs increase in size and accumulate energy throughout spring, summer and autumn (Ansell 1974, Comely 1974, Mackie and Ansell 1993, Sundet and Vahl, 1981, Taylor and Venn 1979). Different reproductive strategies require different use of this storage material versus available food for fueling gametogenesis, as discussed by Ansell (1974), Mathieu and Lubet (1993), and Taylor and Venn (1979). Gonad build up during winter is fueled by stored reserves whereas, gonad build up during spring is fueled by available food. In both cases, the food shortage in winter requires the use of stored energy for maintenance metabolism. Energy storage may also be interrupted by rapid gametogenesis in summer at the expense of energy stored earlier in the season (Barber and Blake 1981, Robinson et al. 1981).

In many populations of *Pecten maximus*, gonads with some degree of fully grown gametes can be found all year, although the gonad indexes vary because of differences in spawning activity and gonad growth (Comely 1974, Mackie and Ansell 1993, Mason 1958, Paulet et al. 1988, Strand and Nylund 1991). Somatic growth and reproduction are competing for resources, and the partition of energy between these two processes is an important trait of the life history of any species. The reproductive effort generally increases with age (Thompson and MacDonald 1991), but the pattern of energy allocation to the gonads within a season has not been given particular attention in studies of scallops.

Better understanding of the processes of growth, storage, and reproduction is a keystone in understanding the general biology of a species and also important for aquaculture, harvest, and management of scallops. This study was conducted with special emphasis on reproduction related to conditioning and spawning in hatchery production. The aim of this study was to describe the variations in weights and content of protein, lipid, and carbohydrates in the female parts of the gonads and in the storage organs during 1 year in a defined population of *P. maximus* in Western

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Norway. Histology and visual characterizations of the female gonads are described elsewhere (Duinker unpublished data).

MATERIALS AND METHODS

Three hundred great scallops (*Pecten maximus*) with shell heights between 95 and 120 mm, shell weights between 90 and 160 g, and ages between 4 and 7 years were obtained from commercial harvest by diving from the area around Bergen. The scallops were individually tagged (Hallprint Pty. Ltd, Australia, ref. no. T1625) and reseeded at the sampling site (N 60 ° 15′ 36″, E 5 ° 05′ 00″) in Raunefjorden south of Bergen on December 11, 1996. The sampling site was a naturally bounded area with shell sand and small gravel at 11–14 m depth. Temperature was recorded by a Tinytag® temperature logger (Intab Interface-Teknik AB, Sweden) attached 50 cm above the seabed at 13 m depth. Samples of 15 scallops were collected by diving and transported in a cooling box with seawater from the site at monthly intervals.

From each scallop, the adductor muscle, digestive gland, gonad, and dried shell were weighed. Samples from the striated adductor muscle, digestive gland, and female part of the gonad were taken for analysis of total glycogen, protein, and lipid. For each organ, samples were pooled into three groups with tissue from five individuals in each and stored at −80 °C.

All samples were freeze dried before analyses (Virtis Genessi 25 SE. Holem, USA), and dry weight percentages were calculated. Glycogen was analyzed using an enzymatic and spectrophotometric method described by Hemre et al. (1989). Protein was analyzed as total nitrogen after total combustion using a Nitrogen-Analyser (Perkin–Elmer, 2410 Ser. II, Norwalk Connecticut, USA). The protein was calculated by the assumption that protein contains 16% N. Lipid was analyzed using a gravimetrical chloroform/methanol method modified from Bligh and Dyer (1959) according to Rønnestad et al. (1995).

The content of glycogen, protein, and lipid in the subsamples were calculated to represent the content per organ as follows: content of constituent = percentage constituent of dry weight × dry weight percentage × average wet weight of the organs in the subsample. Wet weight for the whole hermaphroditic gonad divided by 2 was used for calculating the content of the female gonad constituents, because wet weight was not taken for the female part separately. Hence, the data were not used for evaluating energy flow between the female gonad and the storage organs, but the seasonal variations could still be described. All weights and data for the chemical contents were standardized with shell weight to represent a standard scallop of 100-g shell weight according to the formula: standardized measure = original measure × 100/shell dry weight. Energy from glycogen, protein, and lipid were calculated using conversion factors of 17.38, 23.66, and 35.17 k/g $^{-1}$, respectively (Beukema 1997), and total energy for each organ was calculated as the sum of energy from glycogen, protein, and lipid.

Wet weights were tested for deviations from the normal distribution using the Kolmogorov–Smirnov test (Zar 1999). For the dry weight and chemical content data from the pooled subsamples, normal distribution was assumed according to the Central Limit Theorem (Bhattacharya and Johnson 1977, Zar 1999), because the data were considered as mean values for five individuals. Deviations from homogeneity of variances were tested using the Levene F test (Brown and Forsythe 1974) together with considerations of F-max. Because of pronounced heteroscedasticity in many of the time series, ranks of the data were used in further analyses. Differences between the sampling points were tested with one-way analysis of variance (ANOVA) followed by Newman–Keuls multiple comparison tests when significant differences were found. The results from these tests were compared with results from parametric Newman–Keuls tests and from manually performed nonparametric Newman–Keuls tests (Zar 1999). Statistica version 5.0 (Statsoft inc., Tulsa, OK, USA) was used for all statistical analyses. The significance level (α) was 0.05.

RESULTS

Temperature

Temperature was close to 6 °C from February until the middle of April (Fig. 1). It then increased gradually throughout May and fluctuated between 9 and 12 °C in June, followed by further increases and several sharp drops until the maximum of 19.6 °C in late August. Temperature then dropped to 13 °C in September, gradually decreased until December and then dropped from 8 °C to the winter temperatures of 4 to 6 °C.

Female Gonad

Protein content, lipid content, and dry weight of the female parts of the gonads (Figs. 2 and 3) all followed the same pattern throughout the period of sampling, and the following changes were significant in either one or more of these parameters. Between February 15 and June 8, a decrease was followed by an increase to a new maximal level. A sharp decrease from June 8 was followed by a gradual decrease with stable standard deviations. From minimum levels in September and October, a rapid increase occurred between October 18 and December 16 to maximum levels for the season, and no significant changes in dry weight, protein content, or lipid content were seen on February 7 (Newman–Keuls test on ranks of the data, $0.4 > P > 0.5$).

The glycogen concentration in the female gonads varied between 3 to 5% of dry weight during the period of sampling, and it did not exceed 4% of the average total energy in the female gonad. Protein was the major constituent in the female gonad, with concentrations ranging from 62 to 67% and energy percentages between 67 and 83% during the year. Energy from lipid varied from 29% in February 1997 to 15% in September, corresponding to concentrations between 8 and 19% of dry weight. Maximum con-

Figure. 1. Temperature recordings from January 30 1997 to February 7 1998 at 13 meters depth.

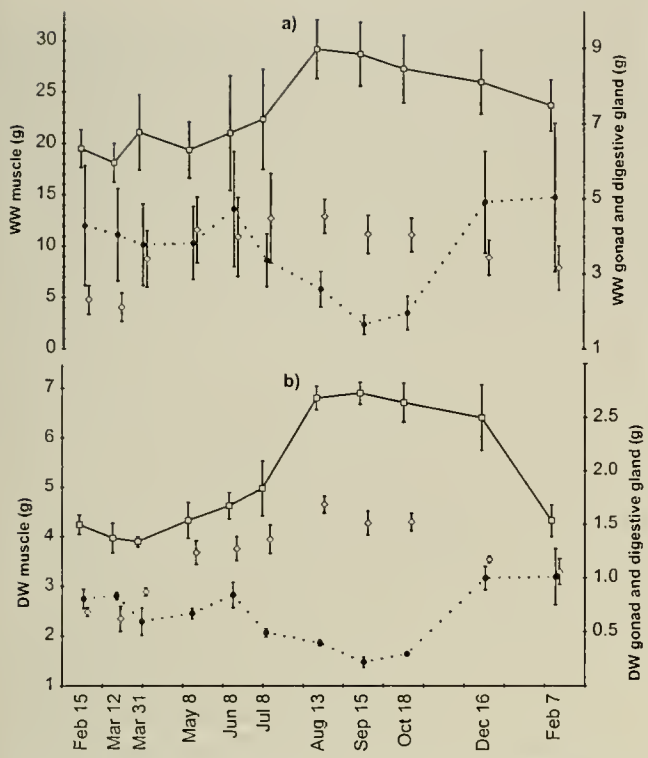

Figure. 2. Seasonal variations in a) wet weight and b) dry weight of the female gonad (---●---), adductor muscle (—□—), and digestive gland (---◇---) for a standardized scallop with a 100 g shell weight. See Materials and Methods for note on the calculation of the female gonad dry weights. Vertical bars indicate standard deviations, $n = 15$ for wet weights, and $n = 3$ from pooled samples for dry weights.

centration of protein and minimum concentration of lipid corresponded with the minimum degree of filling of the gonads in September.

Adductor Muscle

The dry weight (Fig. 2) and content of protein and glycogen (Fig. 3) in the adductor muscles were low at the end of winter with minimum values on March 31. Glycogen increased slowly from March 31 to June 8, with 5.3 mg per day, and then more rapidly until August 13, with 19 mg per day. The increase in glycogen concentration between March and August was from 2 to 25% of dry weight, corresponding to an increase in energy from glycogen from 1.5 to 30 kJ. Protein content was stable with no significant variation until July 3 (Newman–Keuls tests on ranks of the data, $P \leq 0.34$) but then increased rapidly between July 3 and August 13 ($P = 0.01$). Glycogen content decreased from September 15 with a significant decrease between October 18 and December 16 ($P = 0.02$) and a larger decrease from December 16 to February 7 ($P = 0.04$). Protein content was stable between August 13 and February 7 ($P \leq 0.28$).

Lipid content in the adductor muscle (Fig. 3) varied significantly (ANOVA on ranks of the data, $P = 0.006$), but no clear seasonal trends were seen. The lipid level remained low and varied between 3.1 and 4.9% of adductor muscle dry weight.

Digestive Gland

Minimum levels in dry weight, lipid content, and protein content in the digestive glands were found at the end of winter on March 12 (Figs. 2 and 3). Between March 12 and May 8, the protein content increased rapidly with 79% (Newman–Keuls test on ranks of the data, $P < 0.001$). Lipid was accumulated slowly between March 12 and July 3 at 2.8 mg per day, but then increased to 7 mg per day between July 3 and August 13. In August and September, the digestive glands had the maximum levels of lipid for the season, and lipid was the largest constituent in this period. The lipid percentage of dry weight varied from 13.5% on March 12 to 44% on September 15, and energy from lipid varied between 3 and 25 kJ in the same period. Protein content decreased gradually from May 8 to February 7 ($P < 0.001$). Lipid content decreased gradually between September 15 and February 7 ($P = 0.001$). Glycogen remained low and varied between 0.8 and 5% of digestive gland dry weight. Peaks in glycogen content were found in May and in August/September. The loss in total energy from both the adductor muscles and digestive glands between October and December was 11 kJ; whereas, 27 kJ were lost between December and February.

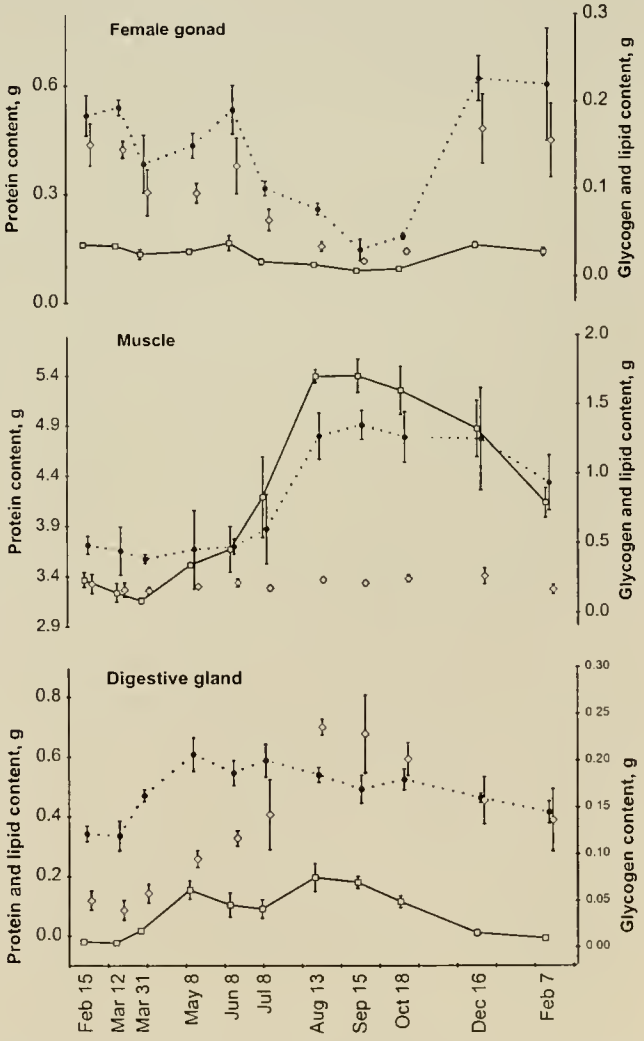

Figure. 3. Seasonal variation in the content of lipid (---◇---), protein (---●---), and glycogen (—□—) in the female gonads, adductor muscles, and digestive glands. See Materials and Methods for note on the calculation of the female gonad data. Values are standardized to a scallop of 100 g dry shell weight. Vertical bars indicate standard deviations, $n = 3$ from pooled samples.

The different multiple comparisons tests that were compared gave largely the same conclusions as to detecting significant differences or not, but the *P*-values varied.

DISCUSSION

Constituents of the Organs

The major constituent of the female gonad was protein, followed by lipid, and glycogen remained low throughout the period of sampling. This agrees with other studies of pectinids (Couturier and Newkirk 1991, Pazos et al. 1997, Taylor and Venn 1979). Female gonads in scallops mainly consist of oocytes and collagen-rich acinus walls (Beninger and Le Pennec 1991), and there are no specialized storage cells as found in other bivalve gonads (Mathieu and Lubet 1993). Hence, the composition of scallop female gonads reflects the composition of bivalve eggs (Gabbot 1976). The protein and lipid content followed the filling of the female gonad, with relatively constant composition despite large variations in filling, which is consistent with the findings of Comely (1974). This probably reflects that growth of the female gonad is propagated by gradual accumulation of increasing numbers of large oocytes. The development of individual eggs is of short duration relative to the period of gonad growth, and several different sized cohorts of oocytes are present at any one time, with recruitment of new cohorts on several points (Paulet and Boucher 1991). A different scenario would be seen if one or a few cohorts were developing synchronously throughout the period of gonad growth, with composition of the female gonad following the development of the individual oocyte. However, at the point of minimum filling of the female gonads in September, the peak in protein percentage and minimum lipid percentage reflect the increasing proportion made up by protein from the acinus walls as the female gonads are emptied.

The adductor muscles consisted mainly of protein but also contained highly variable amounts of glycogen and low lipid levels. Protein represents the major structural part of the scallop adductor muscles, although it can also be utilized to some extent to supply gametogenesis or for maintenance purposes, especially in the semelparous species (Barber and Blake 1991). Protein in the muscles is also the best indicator of somatic growth, that is, increase in size of the whole animal, among the parameters measured in this study (see also Barber and Blake 1981). The fluctuations in glycogen concentration between 2 and 25% are comparable to the 20-fold increase reported in *Pecten maximus* by Comely (1974) and demonstrate the importance of this substrate in energy storage. The importance of glycogen storage in the muscle can be related to the monomyarian condition with reduction in the foot and visceral regions in scallops (Ansell 1974), and to the need for a readily mobilizable carbohydrate reserve for swimming and predator avoidance (Ansell 1974, de Zwaan et al. 1980). The lipid concentration fluctuated between 3 and 5% of dry weight during the sampling period. Giese (1966) concluded that lipid levels less than 5.2 % of dry weight are not to be considered as reserves. The low lipid content in the adductor muscle was, therefore, considered to be structural lipid that made little contribution to the energetics of somatic growth.

In the digestive gland, protein and lipid were the main constituents, with lipid dominating during late summer and protein dominating throughout the rest of the year. Protein represents the enzymes related to the digestive activity of this organ (Beninger and Le Pennec 1991, Henry et al. 1991) and also structural parts. The rapid increase in protein content between March and May coincided with a marked reduction in visibility (own observations), which was attributable to the spring phytoplankton bloom that normally occurs at this time (pers. comm. B. Heimdal, University of Bergen, Norway, see also Erga and Heimdal 1984). The increase in digestive gland protein was probably reflecting an increase in digestive capacity during the spring bloom, a period with high food levels at low temperatures. Hence, it is not regarded as somatic growth in the same context as increase in muscle protein. The decrease in digestive gland protein between May and February may be attributable to reduced needs for high digestive capacity after the spring bloom. A similar increase in digestive gland protein level during the spring bloom occurs in great scallops in the Bay of Brest, France (pers. comm. C. Saout, University of Brest, France). Lipid showed large variations and high maximum levels of more than 40% in late summer. This is consistent with the second role of the digestive gland as the most important site of lipid storage, as suggested from histological studies (Henry et al. 1991) and from losses during gametogenesis (Barber and Blake 1981, Barber and Blake 1991, Robinson et al. 1981). With the low glycogen content, the variations may be attributable to variations in food content in the digestive gland diverticula following variations in feeding activity. The peaks in May and in August/September may correspond to periods with favorable combinations of temperature and food levels giving high feeding rates, and this may reflect conditions similar to those found by Mason (1957) when he reported peaks in filling of the stomachs also in May and August/September.

Because of a low number with the three subsamples, heteroscedasticity in the data, and a high number of groups being compared, the *P*-values from different varieties of the multiple comparison tests were not perfectly consistent. Hence, the statistics alone should not be decisive when considering the seasonal changes, but rather should be used as rough measures of differences between samples related to the variation in the data, in combination with biological considerations. This was preferred to omitting statistics.

Somatic versus Reproductive Growth

The increases in the different constituents observed in this study represent various anabolic processes that can be categorized into: (1) reproductive growth, involving increase in the gonad constituents; (2) somatic growth, represented by increase in adductor muscle protein; (3) increase in digestive capacity, observed as protein increase in the digestive gland; and (4) energy storage with accumulation of glycogen in the adductor muscle and lipid in the digestive gland. The apparently underlying seasonal patterns of these processes suggest a strategy of dividing the year into periods with different priority of reproduction versus somatic growth and storage.

From February to June, gonad growth dominated. Somatic growth was not observed, because adductor muscle protein was stable, and some storage occurred, but at low rates. The scallops displayed an extended period with spawnings of varying intensity between late March and September, and the drops in gonad constituents coincided with evidence of spawning activity (Duinker unpublished data). The net increases and decreases observed in gonad constituents would depend upon the relative contribution of gonad growth versus losses from spawning. Between February and June, it seemed that energy allocation to the gonads was continuous, with rebuilding following the spawnings.

Between June and August, somatic growth and storage domi-

nated, and no increase in gonad constituents was observed. The threefold increase in storage of glycogen in the adductor muscle starting in June suggested that more energy was available for storage from this point. Protein in the muscle showed significant and marked increase only during the narrow period between July and August, with no significant changes during the rest of the year. During the same period, there was also a marked increase in the rate of accumulation of lipid in the digestive gland. Preliminary analyses of daily growth rings were conducted on left valves from the present study according to Chauvaud et al. (1998). This indicated that also shell growth was limited to a period of 3–4 months, depending upon age, that started in June (pers. comm. L. Chauvaud, University of Brest, France). The large drop in gonad content between June and July led to decreased standard deviations and may have been attributable to spawning in large parts of the population. Between July and September, gonad content decreased gradually, with relatively stable standard deviations. This suggests that the gonads were all emptied in steps more or less at the same rate and that no gonads increased in size during this period. Histology showed no signs of massive resorbtion of the oocytes (Duinker unpublished results), so the decrease was probably caused by spawning activity. It seemed that no energy was allocated to the gonads during this period. If correct, this suggests that all available energy was directed to the somatic growth and storage that was observed. Oogenesis seemed to continue as long as the ovaries contained some degree of oocytes of various sizes, but this may have been fueled by recycling of the energy already present in the gonads, in cycles of oocyte growth and atresia (Duinker unpublished results). Verification of such a hypothesis, with filled gonads and ongoing gametogenesis but no input of "new" energy, will cast new light on what seems to be continuous gametogenesis in many populations of *P. maximus*, where filled gonads can be found all the year (Comely 1974, Mason 1958, Paulet et al. 1988, Strand and Nylund 1991, Wilson 1987). Between August and October, neither adductor muscle protein, gonads, or storage increased. A good explanation for this cannot be given. Undoubtedly there was no allocation of energy to the gonads until October. Between October and December, energy was again allocated to the gonads, because the gonads were rebuilt to a new maximum during this period.

Several models developed for plants and animals using dynamic optimization technique have led to the conclusion that somatic and reproductive growth should be separated in time in order to maximize reproductive output, both for semelparous species (Cohen 1971, Mirmirani and Oster 1978, Vincent and Pulliam 1980) and for iteroparous species (Ziolko and Kozlowski 1983). In some situations, however, a model with gradual shifts can be beneficial, although in the end 100% priority of either one of the processes is the result also from such shifts (see discussion in Kozlowski and Ziolko 1988). We suggest that reproductive and somatic growth in the present study were separated in time, with the increase in adductor muscle glycogen storage from June marking a more or less complete shift from reproductive growth to somatic growth and storage and with the start of gonad rebuilding around October marking the shift to again allocating energy to the gonads.

Kozlowski and Uchmanski (1987) assumed instant shifts between reproduction and somatic growth when discussing data from *Chlamys islandica*, although data for the growth pattern within a season were not given in the original data by Vahl (1981). Similar discussions have not been given in studies of other scallop species (e.g., Barber and Blake 1981, Comely 1974, Mackie and Ansell 1993, Pazos et al. 1997, Robinson et al. 1981, Sundet and Lee 1984, Taylor and Venn 1979). However, in a study of *Placopecten magellanicus*, shell and somatic tissue growth took place only in a limited period after spawning (Couturier and Newkirk 1991). Also, the finding of changing and strong negative relationship between RNA/DNA in adductor muscle and gonad during conditioning of *P. magellanicus* (Paon and Kenchington 1995) may reflect similar strategies with separate periods of reproductive and somatic growth. Pearse et al. (1986) suggested for the sea urchin *Strongylocentrotus purpuratus* that the seasonal change in growth and gametogenesis was attributable to competition for energy and resources. Furthermore, they suggested a physiological switch, possibly under photoperiodic control, that determined which process was favored at any one time.

In this study, we did not obtain data on the composition of the male gonad, and it is unknown to us if there were any differences in the relative distribution of energy between the female and male part. Visually, the development of the two parts seemed to follow the same temporal pattern, and there did not seem to be a difference in the timing of growth of the testis and ovary, as has been described for *C. islandica* (Sundet and Lee 1984). However, until proper data have been found, this remains uncertain for *P. maximus*.

Storage and Fueling of Gametogenesis

The decrease in stored energy from September to February coincides with two energy demanding processes: the gonad rebuilding between October and December; and maintenance of the somatic and, eventually, large reproductive tissue. Numerous reports exist on fueling of gonad growth in pectinids by stored reserves and the transfer of substrates from storage organs to the gonad (review by Barber and Blake 1991). In the present study, however, there seemed to be no gonad growth between December and February, and considerably more stored energy, 27 kJ, was lost during this period as compared to the 11 kJ lost during the gonad rebuilding between October and December. This suggests that for the scallops in this study, the gonad rebuilding in autumn was fueled largely by food available during this period and that the more important role of the storage organs is to support maintenance energy demands during winter. Maintenance of a large reproductive mass may be costly as compared to somatic tissue (Bruce 1926). Various strategies for fueling gametogenesis have been reported among pectinids, including gonad rebuilding in winter fueled by stored energy in *P. maximus* (Comely 1974) and *Chlamys opercularis* (Taylor and Venn 1979), gonad growth in spring fueled by available food in *Chlamys septemradiata* (Ansell 1974) and rapid gametogenesis in summer fueled by reserves accumulated the same spring together with available food in *Placopecten magellanicus* (Robinson et al. 1981) and *Argopecten irradians irradians* (Barber and Blake 1981). Furthermore, *P. maximus* from Galicia in Spain displayed two periods of gonad growth, with fueling by stored reserves in winter and by available food in summer (Pazos et al. 1997). In the present study, also the reinitiation of gonad growth in spring was probably fueled by available food, since energy reserves were low and slowly increasing. Hence, these suggestions represent yet another strategy of fueling gonad growth largely by available food, both in autumn and in spring, with a cessation of gonad growth during the period of food shortage in winter. The gonad growth in autumn contrasts with the observation of a resumption of gonad growth in winter in another

study of a population of *P. maximus* south of Bergen (Strand and Nylund 1991). A good explanation for these differences cannot be given at present. Population differences may be possible, but the population structure of *P. maximus* along the coast of Norway is not known, and within the area around Bergen, the exact origin of the scallops used in the present study could not be given by the company that supplied them.

Energy storage and utilization was observed in both the adductor muscles and the digestive glands. Comely (1974) concluded that the adductor muscle was the most important storage organ when considering the energy from both protein and glycogen in *P. maximus*. However, in the present study, adductor muscle protein was stable during autumn and showed only a tendency to decline between December and February, while the other storage substrates decreased from September. This supports the view for some scallop populations that this substrate is preferably not catabolized, and if it is, not until the other substrates are depleted (Barber and Blake 1991). Hence, when comparing the range in energy available from glycogen in the adductor muscle of between 1.5 and 30 kJ with between 3 and 25 kJ for the digestive gland lipid in the present study, the importance of the two storage organs can be regarded as equal. Muscle protein is frequently used, though, and Pazos et al. (1997) found utilization of equal amounts of energy from muscle glycogen, muscle protein, and digestive gland lipid. Considerably more energy was stored during the period of somatic growth in summer than during the period of gonad growth in spring. This may indicate a strategy with controlled somatic growth and opportunistic reproduction as suggested by MacDonald and Thompson (1985) for *P. magellanicus*.

The apparent cessation of gonad growth during winter contrasts with studies of *P. maximus* in the bay of Seine (Lubert et al. 1991) and in the Fosen area in Norway (Strand and Nylund 1991), where gonad growth also started in the autumn but continued throughout the winter. In the present study, both temperature and food may have been limiting factors causing the stoppage. The cessation of gonad growth coincided with observation of clear water (own observations) indicating the reduction to low food levels of winter, at the same time as temperature dropped from 8 to 5 °C. However, it is likely that with elevated food and temperature levels, for example, in hatchery conditioning, gonad growth could have occurred also during this period. A picture then emerges with a seasonal pattern in energy allocation consisting of two parts if food and temperature are not limiting: priority of reproductive growth from about October to June and somatic growth and storage from June to October.

The hypothesis of separate periods of somatic and reproductive growth remains to be tested experimentally, and comparison between populations with differences in their reproductive cycles could provide increased understanding of underlying strategies. As a next step, regulation of timing of the shifts should be investigated. For a given population of scallops, the possible existence of a period without allocation of energy to the gonads would obviously set restrictions on which periods of the year it is possible to induce gonad growth with normal elevated food and temperature conditioning.

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SETTLEMENT SITES OF JUVENILE SCALLOPS *ARGOPECTEN PURPURATUS* (LAMARCK, 1819) IN THE SUBTIDAL ZONE AT PUERTO ALDEA, TONGOY BAY, CHILE

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ABSTRACT A study was made of settlement sites of the Chilean scallop *Argopecten purpuratus* on a small natural bank at Puerto Aldea, Tongoy Bay, Chile (30 °17 'S). Four distinct habitats in the area were surveyed for juvenile scallops, including seagrass, sand-gravel, fine sand with polychaete tubes, and muddy bottom. The highest densities of juvenile *A. purpuratus* were found in areas of fine sand dominated by polychaete tubes (*Diopatra* sp.), followed by areas with sand-gravel and seagrass. No juveniles were found on the muddy bottom. Settlement was found to occur in sites having habitat complexity such as the worm tubes and seagrass, because these provided protective refuge for the scallops, particularly in areas of soft bottom.

KEY WORDS: Chile, scallop, *Argopecten purpuratus*, settlement site, natural bank

INTRODUCTION

Some of the keys to the understanding of population and community dynamics of marine benthic organisms are found in knowledge of the settlement and postsettlement processes of their earliest life stages (Connell 1985, Gaines and Roughgarden 1985, Menge and Sutherland 1976, Menge and Sutherland 1987, Roughgarden et. al. 1985, Rowley 1989, Underwood and Fairweather 1989). This knowledge is of practical importance regarding marine species subject to exploitation or mass culture. Knowledge concerning the intensity of settlement and survival of settled individuals may allow prediction of future population structure, and through this, prognostications on the sustainability of stocks where the resource is exposed to fisheries pressure.

The northern Chilean scallop, *Argopecten purpuratus*, has in the past been exposed to intense exploitation that has depleted its stocks. However, recently it has been cultivated using Japanese technology, which has significantly increased the stock of this species and resulted in the repopulation of natural banks (Stotz in press). A small natural bank at Puerto Aldea on Tongoy Bay showed good recuperation when placed under management by local fishermen and showed potential for sustained exploitation. Data on growth and production of the scallops were obtained from this bank to develop a management plan (Stotz and Gonzalez 1997). However, to understand better the dynamics of the bank and avoid its overexploitation, information concerning the processes of their settlement and recruitment was required. As a first step, data were required concerning the primary settlement sites of the earliest benthic life stages of these scallops.

Information on the settlement process of this species is scarce. Hogg (1977) found small juveniles on the red alga *Rhodymenia* sp. in Herradura Bay, and DiSalvo et al. (1984) mentioned finding a few recently settled postlarvae on ramose bryozoans (*Bugula* sp.) in Tongoy Bay.

In general, pectinids show a strong tendency to settle on a large variety of algae. *Argopecten irradians* juveniles have been associated with algae where they attach to fronds and algal thalli using byssal threads, and thus avoid predation by epibenthic predators (Thayer and Stuart 1974). Juveniles of this species were also observed attached to the seagrasses *Zostera marina* (Eckman 1987, Pohle et al. 1991) and *Halodule wrightii* (Irlandi and Peterson 1991).

Juveniles of other scallop species have also been observed attached to algae. For example, Mason and Drinkwater (1978) found juveniles of *Pecten maximus* and *Chlamys opercularis* byssally attached to *Lithothamnium calcareum*. These data suggest that subtidal areas with high vegetational density are favorable zones for scallop settlement. An example of such a zone in northern Chile is the bed of the seagrass *Heterozostera tasmanica* located off the fishing village of Puerto Aldea.

This bed, measuring about 0.5 by 1 km, has a high density (2,250–4,850 shoots/m^2, Phillips et al. 1983) and is a potential environment for the settlement of juvenile *Argopecten purpuratus* (Gonzalez 1990). The present study recorded the presence of juveniles of *A. purpuratus* in different habitats within the subtidal area around Puerto Aldea and evaluated the importance of the *Heterozostera* bed in the settlement process, relative to sand-, gravel-, and silt-dominated habitats.

MATERIALS AND METHODS

Study Area

The study was carried out near the small fishing village of Puerto Aldea (30 °17 'S, 71 °36 'W) in the SE extremity of Tongoy Bay, about 60 km south of the city of Coquimbo, Chile, SA. (Fig. 1). The physiographic location of Puerto Aldea on the east side of Lengua de Vaca peninsula protects it from prevailing winds and waves, although it may be exposed to (rare) storms from the north. The fishing community of the village has protected the small natural scallop bank (ca. 100 ha) from excessive exploitation since 1991 as a "management area" under Chile's Law of Fisheries and Aquaculture in return for exclusive fishing rights to the bank. The subtidal environment in the area has a sandy to muddy bottom, in which four distinct habitats may be recognized as follows (Fig 2).

Fine Sand with Seagrass

This area occurs in the western part of the management area, extending from the coastline to approximately an 8-m depth, having a bottom consisting of fine sand. Biologically, the community is dominated by the seagrass *Heterozostera tasmanica* in great abundance (2,250–4,850 shoots/m^2, Phillips et al. 1983); the habi-

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Figure. 1. Location of study area at Puerto Aldea, Tongoy Bay.

tat is homogeneous throughout, also showing scattered presence of sand-constructed tubes of the gregarious polychaete *Diopatra* sp.

Sand–Gravel

This sector is located in the central part of the management area, extending from 5–10-m depth, and is characterized by coarse sand and shell particles plus gravel, rock fragments, and boulders to 1.5-m height. Algae characteristic of rocky substrates arise here, including *Dendrymenia* sp., *Cryptomenia obovata*, and *Glossophora kunthii*. In some sectors, there are aggregations of the ascidian *Pyura chilensis*, which may be covered with the red alga *Chondracanthus chamissoi*, which attaches to the ascidian. This alga is also found in small patches on rocks and shells. This habitat was the most heterogeneous of those studied.

Fine Sand with the Polychaetes

This area occupies the SE region of the management area. It extends from the coastline to about a 5-m depth. The sediment is fine sand, with the dominant presence of the tubiculous polychaete species *Diopatra* sp., which produces a generally homogeneous environment including labyrinthine structures providing extensive spatial refuges.

Muddy Sand

This sector was located within the NE part of the management area , extending from 5- to 15-m depth in an ample zone of bare muddy (fine) sand interspersed with small patches of the alga *Sarcodiotheca gaudichaudi* and small clumps of rhodymenial rhodophytes.

Rocks

There are just few rocks in the area, most of which are covered by the ascidian *Pyura chilensis* and/or the red algae *Chondracanthus chamissoi*.

Sampling Design

Timing of Sampling

To determine the best moment for the comparative sampling of habitats, a survey of newly settled juveniles of *Argopecten purpuratus* was carried out between February and May 1997. These months had previously been cited as the setting season for this species in Tongoy Bay (Illanes et al. 1985, Alarcón and Wolff 1991). During these months, every week, five samples of sediment, including flora and fauna, using a 95 cm^2 corer were taken in different, randomly chosen sites within each of the four mayor habitat types cited above (rocks were not included, because they comprise a very small habitat within the area). The presence and abundance of newly settled scallops was verified in each sample. Once the first newly settled juveniles appeared on April 29 in some of the samples signifying the beginning of settlement, the sampling of habitats was scheduled to be started 2 weeks later (May 14) to allow the occurrence of an important amount of settlement before sampling. All the samples were obtained between May 14 and 21.

Sampling Strategy

Because the scale of the natural variability of newly settled juveniles within and between habitats, as well as the scales of variability of environmental characteristics within each habitat, were not known (neither apparent nor obvious), a nested sampling

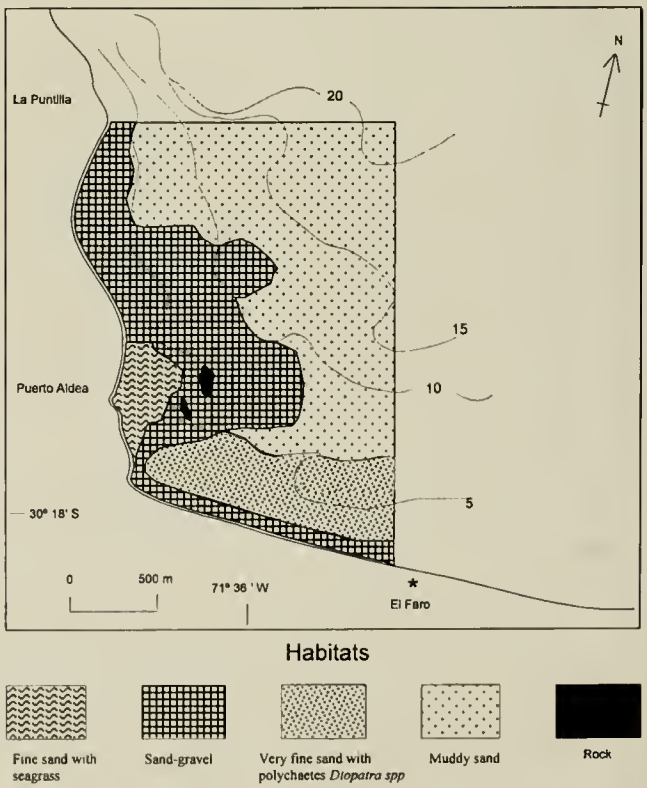


Figure. 2. Habitat types in the study area at Puerto Aldea. Depth contours in meters.

design proposed for such cases by Morrisey and Underwood (1992) was employed, using four spatial scales. This design involved the collection of 108 samples of flora, fauna, and sediment using a 95 cm^2 corer. Sampling was distributed equally on different scales within each of the four habitat types described above. The 27 samples taken within each habitat were taken in such a way that each scale was nested within each larger scale. Thus, each habitat was subdivided into three sites at 100-m distance from each other and then subdivided again into three parcels at 10 m from each other, and again into three replicates at distances of 1 m from each other. The spatial distribution of samples is shown in Fig 3. Dimensions reported for the different sampling scales may not have been obtained with exactitude in practice and represent "best approximations" because of logistical constraints encountered in the fieldwork.

Collection and Analysis of Samples

Samples were collected on 14 and 21 May 1997 by means of HOOKAH diving. A hand-held corer having 95cm^2 area was inserted about 1 cm into the bottom, and sediment was cut off into the corer using a plastic plate. Each sample was then inverted into a plastic bag that had been affixed to the upper end of the corer with an elastic band. The plastic bag was then closed with the elastic band. Samples were returned to shore where they were fixed with 10 % seawater–formalin for subsequent analyses. At the laboratory, samples were washed on a 200-μm mesh nylon screen and then observed in a stereoscopic microscope where the number of juvenile scallops was counted, and shell height was recorded for each specimen.

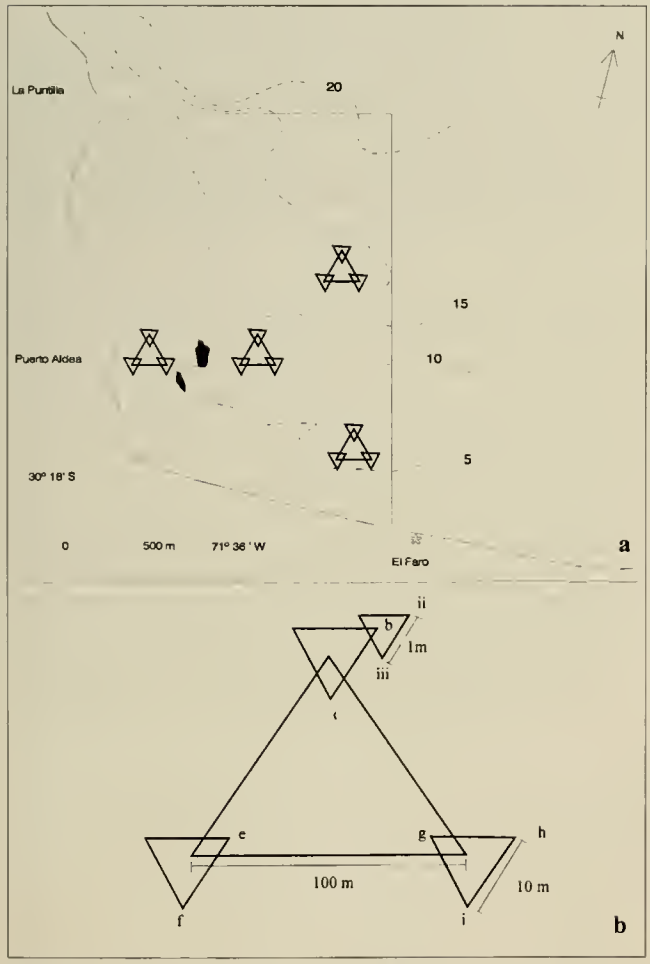


Figure. 3. Distribution of samples (a) between and (b) within the habitats at the study area at Puerto Aldea. Letters represent parcels within the sites and i, ii, iii represent replicates within the parcels. Depth contours in meters.

Statistical Analysis

Given the heterogeneity of the variances, all data were transformed using log (X + 1) and then a nested analysis of variance (ANOVA) was used to compare the abundance of scallop juveniles among the distinct habitats sampled, among sites within habitats, and among plots within sites. This analysis permitted calculation of the amount of total variation in abundance given by each sampling scale with the total variation of abundance (Morrisey and Underwood 1992). The nested ANOVA is more robust and powerful when the sampling design is balanced; that is, when each level of a factor (scale) has the same number of replicates within it (Morrisey and Underwood 1992). In this way, it was possible to determine if the potential differences encountered along the scale of habitats was attributable to intrinsic factors in each habitat or to the contribution from variances within the smaller scales.

To establish the pattern of spatial distribution of newly settled juveniles, a chi-square analysis was performed, comparing their distribution with a Poisson and negative binomial distribution.

RESULTS

Desciption of Juveniles

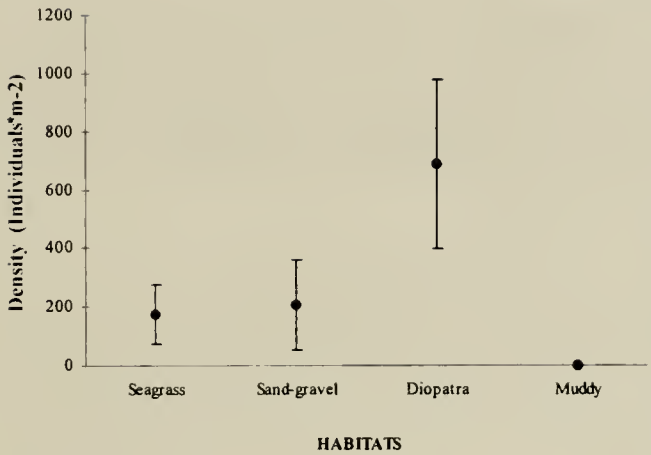
Juveniles smaller than 1.53 mm shell height had uniformly white shells with concentric striae; both valves had circles of dark color on their dorsal sector. The right valve was smaller in size than the left valve, with the latter more concave than the former. Juveniles larger than 1.53 mm shell height had violet colored shells and had both radial and concentric striae. The size of the valves was similar but with the left valve continuing to be more concave than the right; although, this difference was less notable than in the small individuals. The shells of these small specimens begin to resemble adult shells at a very early stage.

Timing of Sampling

No juvenile *Argopecten purpuratus* were found in the study area during the first 11 weeks of observation; recently settled juveniles were first observed on 29 April 1997. These first individuals were found only in the sand–gravel sector at low densities (4.6 ± 2.41 individuals m^{-2}). Sizes of juveniles recovered from this area in that moment varied between 0.306 and 4.173 mm in height. Thus, when the comparative sampling of habitats took place, settlement had been occurring for at least 2 weeks before sampling. It is assumed that, considering the small distances between habitats compared to water movement (several m sec^{-2}), within this time larvae may have equally reached all sites within the study area.

Patterns of Abundance

Settlement occurred in only three of the four habitats analyzed. There was no settlement observed in the muddy-sand sector. The highest density of juveniles (686 individuals m^{-2}) was found in the habitat dominated by *Diopatra* sp. tubes followed by the sand–gravel sector (206 individuals m^{-2}), and finally the seagrass sector,



q	Sand-gravel	Diopatra	Muddy
Seagrass	0,15 ns	2,42 ***	0,82 ns
Sand-gravel		2,27 ***	0,98 ns
Diopatra			3,25 ***

Figure 4. Mean density of juvenile *Argopecten purpuratus* in different habitats at Puerto Aldea. Table gives the results of Tukey's test on pairs. ***significant difference, ns = no significant difference.

with 174 individual m^{-2} (Fig 4). The ANOVA showed a significant difference among the habitats, with no significant differences among sites within habitats or among plots within sites (Table 1). The power of the analysis was 0.97 with a $\alpha = 0.01$. This result gave statistical support to the hypothesis that differences among habitats were attributable to intrinsic characteristics of each and not to random variation or to variation among sites within habitats or among plots within sites. Despite this finding, high total percentage variation (38.7%) was observed at the 1- m scale. It was shown, using Tukey's test, that abundance of scallop juveniles in the *Diopatra* sp.-dominated habitat was significantly higher than in the other habitats sampled. There were no significant differences between sand–gravel and seagrass areas.

Size Structure

The smallest scallop juveniles were found in the *Diopatra* sp.-dominated habitat (Fig. 5). The sizes of the juvenile scallops from all habitats fluctuated from 0.350 to 6.535 mm in height (size classes from 0.25 to 6.75 mm). A size/frequency histogram showed the most abundant size class to be that measuring 0.75 mm and 87% of the sampled individuals measured less than 1.75 mm in height (Fig 6).

Patterns of Spatial Distribution

The distribution of juvenile scallops in the bank was significantly different from random (Poisson), and resembled a negative binomial distribution (χ^2: 24.3, degrees of freedom (dof).: 7, $P > 0.001$). This observation was supported when utilizing the Morisita index, which suggested a pattern of aggregated distribution (Table 2). Analysis of distribution of recently set scallops by habitat type produced a negative binomial distribution; that is, aggregated distributions within each different habitat (seagrass: χ^2: 11.02, dof: 20, $P > 0.05$; sand–gravel: χ^2: 25, dof: 20, $P > 0.05$; Diopatra: χ^2: 29, dof: 20, $P > 0.05$). The degree of aggregation is similar for the different habitat types, but the different habitats show different degrees of environmental heterogeneity. For example, the sand–gravel habitat is much more heterogeneous than the other habitat types. This suggests that the aggregation of scallops is an attribute attributable to a behavior of the scallops, not an attribute of the habitat. It is probable that new arrivals settle close to individuals already settled.

DISCUSSION

Larvae of *A. purpuratus*, cultivated in the laboratory at 14 °C, reached metamorphosis after 30 days of culture, at a length of about 231 ± 10 μm (Bellolio et al. 1993). In the laboratory, postlarvae were observed in 30 days to attain about the same size as the smallest specimens obtained by us at the Puerto Aldea site (< 1.75 mm). Once settled, juveniles of *A. purpuratus* remained attached to settlement sites by their byssal threads until they reached 8–10 mm in height (Navarro et al. 1991). A similar size (11 mm) was noted for in *A. irradians* (Garcia-Ezquivel and Bricelj 1993). Based on these observations and the small sizes of the juvenile scallops found in our sampling (< 7 mm shell height), it is probable that these juveniles had remained at their original sites of settlement. It was, therefore, assumed that the pattern of abundance and distribution of juveniles collected in the present study reflected the natural settlement pattern and could be used as an indirect measure of this, as suggested by Rowley (1989). Thus, prevalence of juvenile *A. purpuratus* in habitats dominated by *Diopatra* sp. tubes, the seagrass *Heterozostera tasmanica*, and in the sand–gravel area reflect preference for initial settlement in these areas. No settlement was recorded for the muddy bottom in our study, as noted for other Pectinid species that fail to settle on this type of bottom (e.g., *Chlamys varia, Pecten maximus, A. opercularis*; Burnell 1991). Although settlement may have occurred on this substrate, followed by mortality, no empty shells were encountered in our sampling,

TABLE 1.

Analysis of variance of the abundance of newly settled juveniles of *Argopecten purpuratus* in different habitats at Puerto Aldea (Chile) (significance level at $P < 0.001$).

Source of Variation	Degrees of Freedom	S.S.	M.S.	F_s	%
Between habitats	3	92.70	30.90	19.6***	48.65%
Between sites within habitats	8	12.62	1.58	3.9 ns	5.84%
Between plots within sites	24	9.70	0.40	0.47 ns	6.85%
Error	72	62.15	0.86		38.66%
Total	107	177.18			

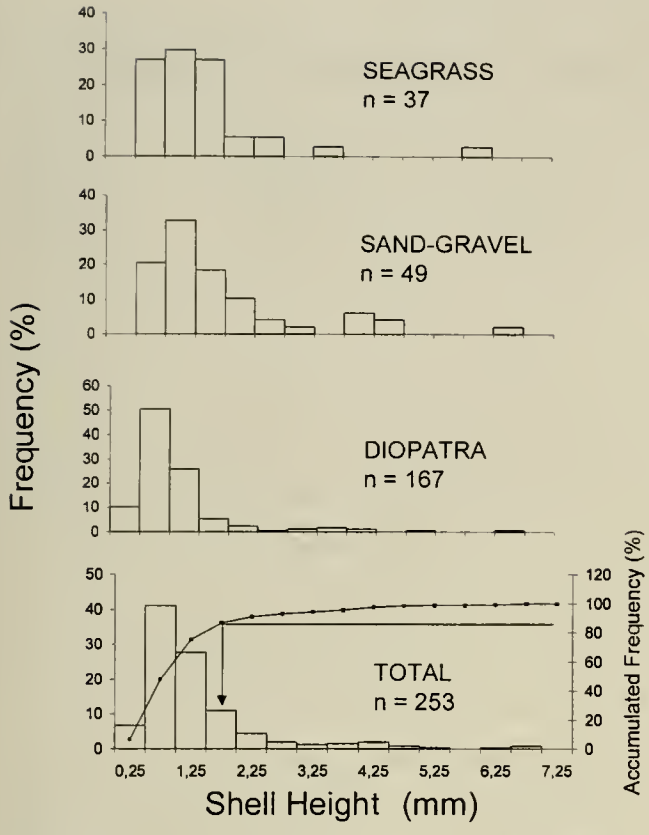


Figure. 5. Size structure of juvenile *A. purpuratus* in each habitat. Size structure of all sampled juvenile *A. purpuratus* at Puerto Aldea (n = 253) and cumulative frequencies showing the class-mark (arrow) at which 85 % of the individuals are included.

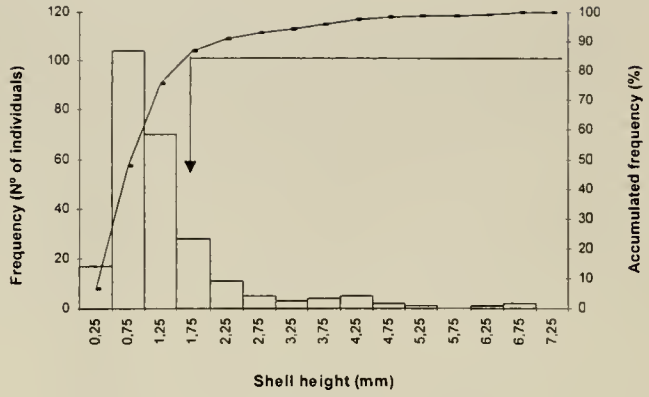


Figure 6.

which would support such a hypothesis. Because this habitat is at a greater depth (10–15 m depth) as compared to the others, the larval supply may have been poor, because larvae have been shown to prefer settlement at lesser depths.

The habitats in which settlement was found to occur have in common an erect ministructure that may provide important refugia for scallop settlement, particularly over soft bottoms. This has been cited for diverse species of pectinids, where settlement has been reported on algae (Ambrose and Irlandi 1992, Mason and Drinkwater 1978, Minchin 1976), sea grasses (Eckman 1987, Irlandi and Peterson 1991, Pohle et al. 1991, Thayer and Stuart 1974), such metazoans as bryozoans and hydroids (Allen 1979), and extensive metazoan-produced substrates such as polychaete tubes (*Sabella pavonina*) or ascidian tunic (*Ascidiella aspersa*) cited by Burnell (1991). Settlement on erect structures favors the growth of the scallop juveniles, because they are exposed to more water movement than near the seabed, which gives them a better quality diet (Eckman and Peterson 1989). Moreover, their elevated position allows them to evade predation by epibenthic predators (Thayer and Stuart 1974) as well as to avoid burial in soft sediments (Ambrose and Irlandi 1992, Pohle et al. 1991). The complexity of the habitat afforded by the erect structures may also afford some protection from predation, as found by Pohle et al. (1991), who demonstrated a direct relation between survival of *A. irradians* and the density of stems of *Zostera marina*; a similar relationship was demonstrated for several species between density of algae or seagrass and loss to predation. For example, a decrease in foraging behavior of the pinfish *Lagodon rhomboides* has been observed as an effect of the presence of benthic macrophytes (Stoner 1982, Main 1987). Similarly, inhibition of foraging by macrophytes was also observed in *Penaeus duorarum*, fishes, and decapods (Leber 1985), as well as for amphipods living associated to algae (Nelson 1979). The major occurrence of settlement of *A. purpuratus* over a soft bottom inhabited by *Dioptra* sp. tubes may indicate a preference for a less complex habitat than the *Heterozostera* stems in favor of one with more permanence. Seagrass stems undergo continual death and renovation, increasing the probability of loss of the scallops (Eckman 1987, Pohle et al. 1991). The *Diopatra* sp. tubes, constructed of sand and shell fragments may represent habitat more secure from perturbation (Woodin 1978). In addition, water flow over the *Diopatra* tubes may be better than within a dense seagrass meadow or algal bed. The tubes produce little resistance to water flow, because they emerge only slightly from the bottom. Moreover, selective adaptation for the *Diopatra* sp. microhabitat is more probable, because the occurrence of *Heterozostera* in Chile is unusual, with the bed at Puerto Aldea, and a second smaller one farther north, being its only known occurrence on this coastline (Phillips et al. 1983, Gonzalez and Edding 1990).

Settlement of scallops on the algae *Chondracanthus chamissoi* in the study area remains problematical, because local fishermen harvest this alga commercially in spring and summer. If scallop settlement should occur in periods before the algae harvest, important mortality of juvenile scallops may occur. Thus, it is important to carry out studies in the future that provide data useful in minimizing this potential impact on juvenile scallops.

The pattern of distribution of juvenile scallops at Puerto Aldea suggested the occurrence of aggregated (patchy) settlement. Although the habitats were different in their degrees of complexity, with the sand–gravel areas more heterogeneous than the others, the degree of distribution of scallops within the distinct habitats was similar. This fact suggested that aggregated settlement was a property of the species, wherein the settlement of some individuals induced further settlement by their congeners.

The areas containing the juveniles were not correlated with sectors containing high densities of adults (Stotz and Gonzalez 1997). Differential mortality may have occurred between habitats as observed by Luckenbach (1984) for an estuarine bivalve. Because detached scallops are active swimmers, they probably distribute themselves to habitats most favorably suited to their growth and survival. This suggests that postsettlement processes were oc-

TABLE 2.

Chi-square analysis comparing the distribution observed for *A. purpuratus* juveniles with a negative binomial and Poisson distributions using data from 105 cores obtained in the study area. (***) expected frequency significant at $P < 0.001$; ns = nonsignificant.

Negative Binomial Distribution			Poisson Distribution		
No Scallops by Core	Observed Frequency	Expected Frequency	No Scallops by Core	Observed Frequency	Expected Frequency
0	47	47.00	0	47	8.04
1	12	16.33	1	12	20.65
2	15	9.90	2	15	26.54
3	6	6.85	3	6	22.74
4	3	5.04	4	3	14.61
5	6	3.84	5	6	7.51
6	6	2.99	6	6	3.22
7 and more	10	12.18	7 and more	10	1.70
χ^2		9.36 ns			262.19***

Mean: 2.57
Variance: 16.56
No samples: 105
No scallops: 270
$\Sigma\chi^2$: 2412
K: 0.4016447 to negative binomial
Morisita index (Im): 3.1

curring that later determined the distribution of the adults as suggested by Rowley (1989).

ACKNOWLEDGMENTS

The authors are grateful to the fishermen's organization of Puerto Aldea for the facilities given during the fieldwork. special recognition to Sergio González, who helped with the diving and collection of samples, and to Domingo Lancellotti who helped with the statistic design. Thanks are given also to Louis DiSalvo, who translated the paper and provided editorial advice. Financial support was partially received by the Regional Government, through project BIP 20065131.

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A COMPARISON OF SIZE SELECTIVITY AND RELATIVE EFFICIENCY OF SEA SCALLOP, *PLACOPECTEN MAGELLANICUS* (GMELIN, 1791), TRAWLS AND DREDGES

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ABSTRACT During August and September 1997 and May 1998, three comparative fishing experiments were conducted aboard commercial sea scallop trawl and dredge vessels to assess the efficacy of gear restrictions found in Amendment 4 to the Sea Scallop Fishery Management Plan (SSFMP). This amendment involved certain gear restrictions including minimum mesh and ring sizes and maximum gear widths and was intended to equate the performance of sea scallop trawls and dredges with respect to size selectivity and efficiency. Statistical analysis indicated that selectivity and efficiency were not equal for the two gear types. While absolute gear size selectivity could not be estimated, there was clear evidence of differential relative size selectivity between the two gears. Relative harvest efficiency values shifted at 90 to 95 mm shell height. Trawl vessels were more efficient capturing sea scallops less than 90 mm, and dredge vessels were more efficient capturing sea scallops greater than 90 mm. This shift in relative harvest efficiency coupled with an observed cull size at 70 to 75 mm shell height resulted in the trawl vessels being more dependent on age 3 sea scallops with shell heights of 70 to 90 mm. Operational differences observed between the two gear types restricted sea scallop trawl vessels to areas of smooth substrate. Large differences in both relative efficiencies and operational requirements will present considerable impediments to the desired outcomes of having equivalent performance between gear types.

KEY WORDS: sea scallops, *Placopecten magellanicus*, fishing gear, relative efficiency

INTRODUCTION

Wild populations of the sea scallop, *Placopecten magellanicus*, occur exclusively on the continental shelf of the northwestern Atlantic Ocean from the Canadian Maritimes to Cape Hatteras, North Carolina (Posgay 1957). Within the Exclusive Economic Zone (EEZ) of the United States, the commercial sea scallop fleet is comprised of vessels using both dredges and modified otter trawls. During 1998, dredge vessels operating coastwide, accounted for 90% of total landings, while trawl vessels focused operations on the softer substrates of the mid-Atlantic resource area tallied the remaining 10%. Total sea scallop landings for 1998 were 5,549 metric tons of shucked meats valued at $74.8 million (NEFMC 1999).

Sea scallop landings peaked in 1990 when a record high 17,500 metric tons of shucked meats worth $149 million were landed (NEFMC 1999). The sea scallop fishery, however, has historically been characterized by cycles of high and low production due to fluctuations in recruitment and varying levels of fishing effort (Dickie 1955). The onset of more frequent and extreme fluctuations in landings during the late 1960s and early 1970s, coupled with dramatic increases in ex-vessel prices, effort, and capital prompted federal regulatory measures (NEFMC 1982). Since May 1982, the sea scallop fishery was managed under the provisions found in the Sea Scallop Fishery Management Plan (SSFMP).

Regulatory measures found in the SSFMP initially focused on controlling age at entry in an effort to maximize yield per recruit (NEFMC 1982). Regulations required an average meat count for shucked scallop meats and a minimum size for shell-stocked sea scallops (sea scallops landed in the shell). These regulations, however, proved to be inadequate and resulted in the continued exploitation of small sea scallops (>40 meats per pound, MPP), high levels of fishing mortality (F), and allegations of inequity between dredge and trawl vessels (Naidu 1987, Shumway and Schick 1987, DuPaul et al. 1989b, 1990, Kirkley and DuPaul 1989, Schmitzer et al. 1991). To address these problems, Amendment 4 to the SSFMP, adopted in 1994, changed the management strategy to an effort control program in an attempt to reduce F by 70% over a 7 year rebuilding period (NEFMC 1993). The primary measures of Amendment Number 4 included the establishment of a limited access fishery and the institution of days at sea restrictions (NEFMC 1993). Supplemental measures included gear restrictions, crew size limits, vessel replacement restrictions, and catch limits for non-permitted vessels (NEFMC 1993).

Although the management strategy was changed by Amendment 4, the objective of establishing age at entry was again addressed. Modifications to the two gear types in the fishery replaced the meat count and shell height restrictions in an attempt to control age at entry. These modifications would theoretically allow juvenile sea scallops (<70-mm shell height) to escape the gear, rather than relying on the crew to discard them. Sea scallop dredges were required to meet specific criteria of ring size, chafing gear, twine tops, and maximum dredge width. The configuration of sea scallop otter trawls were restricted on the basis of minimum mesh size, mesh orientation, and maximum trawl sweep.

The gear restrictions found in Amendment 4 were guided by the assumption that these modifications would result in equivalent performance between trawls and dredges with respect to size selectivity and harvest efficiency. Equivalent performance of the two gear types addresses a management objective attempting to control sea scallop age at first capture and a policy mandate that requires equity between user groups. There are no data to support the assumption that Amendment 4 gear restrictions would achieve the desired result. Comparisons of sea scallop dredge and trawl gear have been conducted by Kirkley (1986) and DuPaul et al. (1989c), however, the gear consisted of smaller mesh and ring dimensions than required by Amendment 4.

The objective of this study was to examine size selectivity and relative efficiency of sea scallop trawls and dredges as regulated under Amendment 4 to the SSFMP. This comparison will establish whether Amendment 4 gear restrictions are effective in both controlling sea scallop age at entry to the fishery.

and results in the equitable treatment of user groups operating in the U.S. sea scallop fleet.

MATERIALS AND METHODS

The study area was located along the continental shelf off the East Coast of the United States from Sandy Hook, New Jersey to the Virginia/North Carolina border (Figure 1). Water depths in the study area ranged from 25 to 45 fathoms (46–82 m). Seabed topography and substrate composition were uniform throughout the area, dominated by level expanses of mud and sand with scattered areas of large boulders. This general area is considered a traditional sea scallop fishing ground, however, specific areas for the comparative fishing experiments were located using the local knowledge of the participating commercial vessel captains.

Gear deployment and vessel design constraints prevented a dredge and an otter trawl from being towed by the same vessel simultaneously. The comparison of the two gear types was conducted by sampling with both a commercial dredge vessel and a commercial otter trawl vessel. Utilizing the parallel fishing method the two vessels fished the same ground at the same time and sampled from a single population of sea scallops (Pope et al. 1975). To ensure that the criteria of the sampling design was met, data from tows which were sampled, but did not occur in the same area at the same time were subsequently excluded from analysis. The study consisted of three comparative fishing cruises conducted as an adjunct to normal commercial fishing trips between August 1997 and May 1998. The only modification to a commercial fishing trip being that both vessels operate in the same area at the same time and use Amendment 4 compliant fishing gear.

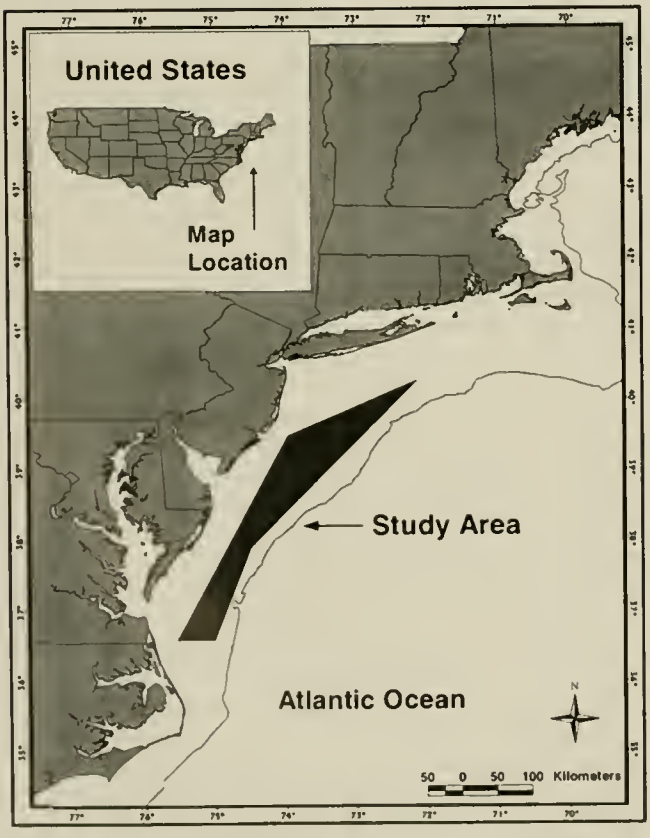

Figure 1. Map depicting the location of the three comparative trips.

A description of the New Bedford style offshore sea scallop dredge is given by Posgay (1957) and Bourne (1964). Pursuant to Amendment 4 restrictions, the chain bags of all dredges were knit with rings that had an inside diameter no greater than 3.50″ (89 mm). Standard 5.50″ (140 mm) diamond mesh twine tops were used on all dredges, and split tire shingles were used on the bottom of the chain bags as chafing gear.

The sea scallop otter trawl vessels utilized two trawls towed from separate warps. Wood trawl doors with dimensions of 120″ × 40″ (3.05 × 1.01 m) were attached directly to the wings of the nets. Steel sleds (approximately 400 lb. [181 kg]) in place of trawl doors were used on the inner wings of the two nets. The bodies and codend of the trawls consisted of 5.50″ (140 mm) diamond mesh. Varying configurations of sweep chains ranging from 1/2″ (12.7 mm) to 5/8″ (15.9 mm) were used on the footropes of the trawls. A 1/2″ (12.7 mm) tickler chain was also used. Chafing gear consisted of a doubled 1-m piece of nylon attached to each mesh on the belly of the codend. The length of warp fished varied with depth, but generally was held at a warp length/depth ratio of 3:1.

Deck operations were conducted under near normal commercial fishing conditions. For all tows, the catch from each gear was dumped on the deck, culled, shucked, bagged, and placed on ice or frozen until the termination of the trip. For comparative tows that were sampled, the crew culled the catch for sea scallops to be retained for shucking. A subsample of up to two baskets (1 basket equals approximately 1.5 bushels [53 L]) of retained sea scallops were set aside for length frequency analysis. Discarded sea scallops were subsampled as appropriate depending on the volume of trash and number of discards present. A shell height for each sampled scallop was taken in 5 mm intervals from the umbo to the ventral margin of the shell using a NMFS sea scallop measuring board.

Catch data were standardized to reflect harvest per unit area covered by the fishing gear. Linear distance traveled for each tow was calculated as the product of towing speed and tow duration. Area swept for each tow was estimated as the product of linear distance traveled and gear width. Dredge width varied between trips and was either 14 ft. (4.6 m) or 15 ft. (4.5 m). Trawl mouth spread was calculated as one-half the average of the headrope and the footrope (DeAlteris 1998). Kostyunin (1971) reported the fishing spread of modern trawl nets to be from 45% to 50% of the headline length corroborates this estimate. The estimates of area swept by the gear were then converted to hectares (1 ha = 10,000 m^2).

Relative harvest efficiency was calculated as the percentage difference in the number of sea scallops captured per hectare by the trawl relative to the dredge for each shell height size class. Relative production efficiency was examined with respect to the number of sea scallops harvested, production of scallop meats (grams), and average MPP at both observed cull sizes and at hypothetical cull sizes of 70, 80, and 90 mm shell heights. To estimate production of scallop meats and MPP, a shell height:meat weight allometric relationship for the mid-Atlantic region was applied to the midpoints of the shell height intervals (NEFMC 1982):

$$W = 5.929 \times 10^{-6} L^{3.234},$$

L = shell height and W = meat weight. Statistical differences in mean number of sea scallops harvested, mean production rates, and average MPP between the gear types were determined by a two tailed Student's t test at the 5% significance level.

Size selectivity in the sea scallop fishery occurs as two different

TABLE 1.

Summary of operational procedures for comparative gear trials.

	Trip 1		Trip 2		Trip 3	
Date	August 8 through 18, 1997		September 8 through 18, 1997		May 13 through 18, 1998	
Area	Virginia Beach, VA		Hudson Canyon		Chincoteague, VA	
Vessel	Stephanie B.	Triangle I	Carolina Breeze	Capt. AT	Carolina Clipper	Triangle I
Gear	Dredge	Trawl	Dredge	Trawl	Dredge	Trawl
Tows on trip	199	80	286	99	121	48
Comparative tows	77	34	49	30	29	14
Scallops measured	31,689	47,385	13,685	22,665	24,455	24,929

processes: that imposed by the type and characteristics of the fishing gear and that imposed by the crew culling the catch. Estimates of relative size selectivity and efficiency were inferred for the two gear types from the numbers of sea scallops harvested and shell height frequency distributions. The size selection characteristics of the crew were determined by collecting the data in a manner that differentiated between sea scallops that were retained for shucking or discarded. The crew size selection curve was calculated as the ratio of the number of sea scallops retained by the crew for shucking to the total number of sea scallops captured for each shell height. Linear regression of normal deviates versus shell height was performed to determine the 25%, 50%, 75%, and 100% retention shell heights and selection range. Selection range was defined as the difference between the 75% and 25% retention shell heights.

RESULTS

Trip Data

Data for the study was collected on three comparative fishing trips during August and September of 1997 and May of 1998. Each comparative trip was considered an individual set of trials due to differences in geographic location and sea scallop abundance and size composition. Operational procedures for each set of trials are shown in Table 1. Sea scallop shell height frequencies for each individual trip are shown in Figure 2.

Crew Size Selection

The estimated selectivity parameters for sea scallops retained by the crew for shucking with associated size selectivity curves are shown in Table 2 and Figure 3. Although the size composition of the target species varied considerably over the three trips, crew size selection remained relatively constant. The shell height at which a scallop had a 50% chance of being retained for shucking (L_{50}) ranged from 69.3 to 77.5 mm. Scallop sizes from L_{75} to L_{25} ranged from 3.6 to 12.0 mm, which indicated that the crew selection process was relatively knife edged. Size selection of sea scallops was complete (L_{100}) at shell heights that ranged from 79.4 to 109.7 mm. However, larger sea scallops (>90 mm) classified as discards were probably the result of oversights by the crew.

Relative Efficiency

Relative harvest efficiency for each trip is shown in Figure 4. The relative harvest efficiencies of the gear types were approximately equal at a shell height range of 85 to 95 mm. Sea scallop catch per unit effort at a shell heights of 85 to 95 mm were not statistically different ($P > 0.05$) between gears for all three trips. Trawl vessels harvested sea scallops less than 85 to 95 mm shell height more efficiently and sea scallops greater than 85 to 95 mm shell height less efficiently relative to the dredge vessels. Relative harvest efficiency values for small sea scallops (<30 mm shell height) and large sea scallops (>130 mm shell height) meant little as sample sizes were limited.

Catch statistics for each trip calculated using the observed culling practices of the crew are shown in Table 3. Length frequency distributions for sea scallops taken by dredges and trawls vessels differed appreciably. However, the total number of sea scallops harvested and retained per hectare swept by the trawl gear was not statistically significant ($\alpha = 0.05$). Greater numbers of larger sea

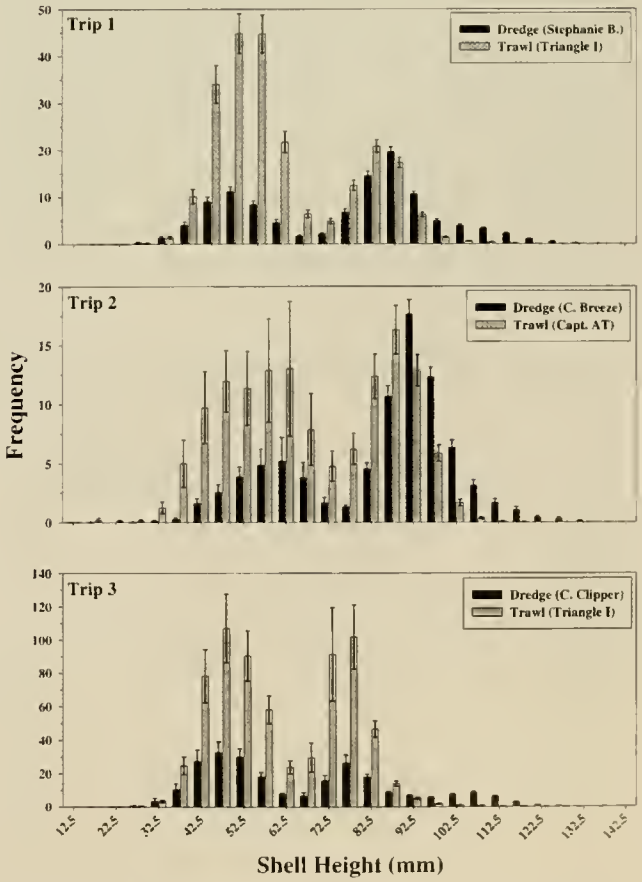

Figure 2. Shell height frequency distributions (mean ± SE) for each comparative trip standardized to one hectare covered by the gear.

TABLE 2.

Crew size selection lengths for all comparative gear trips. Values represent shell heights in millimeters at which a scallop had a 25%, 50%, 75%, and 100% probability of being retained by the crew for shucking.

	Trip 1 (August 1997)		Trip 2 (September 1997)		Trip 3 (May 1998	
	Stephanie B. Dredge	Triangle I Trawl	C. Breeze Dredge	Capt. AT Trawl	C. Clipper Dredge	Triangle I Trawl
Selection lengths						
L_{25}	73.0	71.8	67.5	68.0	70.5	74.3
L_{50}	76.5	75.9	69.3	71.6	76.5	77.5
L_{75}	80.0	80.0	71.1	75.2	82.5	80.6
L_{100}	95.9	98.6	79.4	91.3	109.7	94.9
Selection range L_{75}–L_{25}	7.0	8.2	3.6	7.2	12.0	6.3

scallops with larger meats harvested by the dredge vessel resulted in significantly higher ($P < 0.05$) production rates during August 1997. Differences in production rates for September 1997 and May 1998 were not statistically significant at the 5% level. MPP from the trawl vessels were significantly higher ($P < 0.05$) than meat counts from the dredge vessels for all trips.

The trawl vessel on the May 1998 trip took 35.4% more sea scallops per hectare than the dredge vessel. This difference was due to large numbers of 70 to 90 mm shell height sea scallops which constituted 92% and 58% of the catches of the trawl and dredge boats, respectively. Larger meats from the greater numbers of 90+ mm sea scallops captured by the dredge boat, however, resulted in the trawl boat being 8% less efficient relative to the dredge boat with respect to grams of meats produced per hectare.

During the August 1997 and September 1997 trips, 70 to 90 mm sea scallops were less abundant. Sea scallops in this size range constituted 57% and 62% of the catch by the trawl boats and 32% and 28% of the dredge boats for the August 1997 and September 1997 trips, respectively. Trawl boats on these two sampling trips were 6.5% and 0.7% less efficient than those using dredges with respect to the number of sea scallops caught per hectare due to the paucity of 70 to 90 mm sea scallops. Differences in the number of large sea scallops harvested resulted in the trawl boats being 27.4% and 25.3% less efficient relative to the dredge boats with respect to grams of scallop meats produced per hectare.

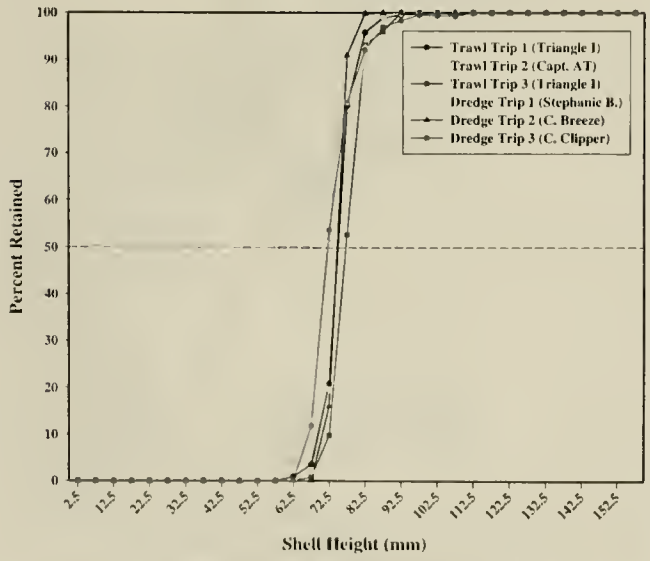

Figure 3. Size selection curves for the crew culling process.

Relative Efficiency at 70, 80, and 90 mm Shell Heights

Relative production efficiency was also examined by imposing hypothetical culling sizes of 70, 80, and 90 mm shell heights to examine the effects of possible changes in scallop age at entry to the fishery. This analysis further demonstrated the effect that differential catch compositions had on the comparison between the

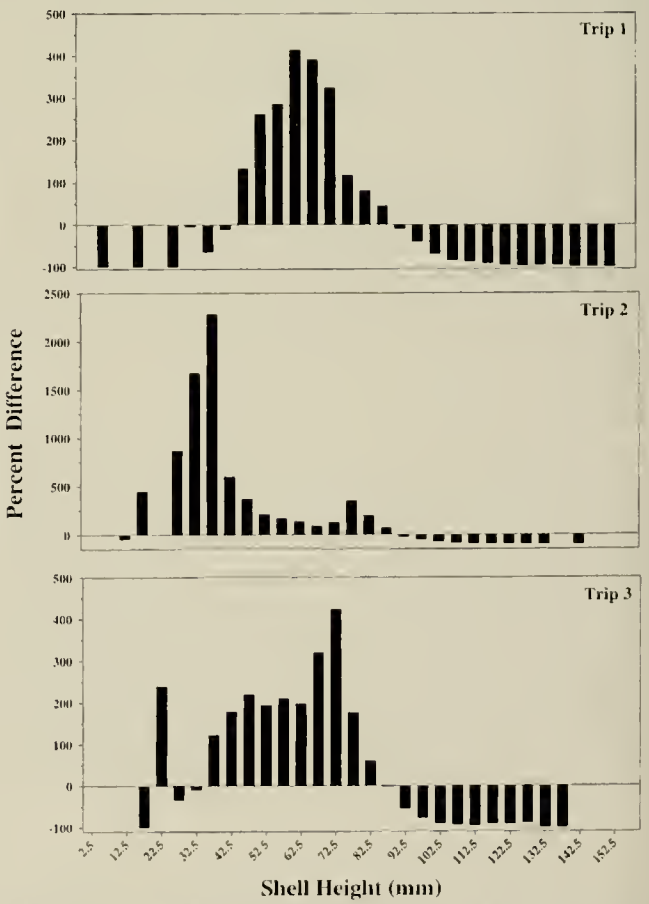

Figure 4. Relative harvest efficiency of the 5.50″ (140 mm) diamond mesh sea scallop otter trawl relative to the 3.50″ (89 mm) ring sea scallop dredge for all comparative trips.

TABLE 3.

Mean number of sea scallops harvested, mean grams of scallop meats produced, and average meats per pound (MPP) for all comparative gear trips. Values were calculated using the observed culling practices of the crew with the data standardized to reflect catch per hectare covered by the gear.

	Trip 1 (August 1997)		Trip 2 (September 1997)		Trip 3 (May 1998)	
	Stephanie B. Dredge (n = 34)	Triangle 1 Trawl (n = 77)	C. Breeze Dredge (n = 30)	Capt. AT Trawl (n = 49)	C. Clipper Dredge (n = 29)	Triangle 1 Trawl (n = 14)
Harvest (#/ha.)	69.0 ± 2.4	64.5 ± 3.7	59.9 ± 2.8	59.5 ± 5.5	96.4 ± 7.7	130.5 ± 17.3
Production (grams/ha.)	1,068.4 ± 33.5*	776.1 ± 42.6*	908.5 ± 44.8	687.9 ± 61.9	1,298.0 ± 73.4	1,194.2 ± 141.9
MPP	35.6 ± 0.4*	44.4 ± 0.6*	35.9 ± 0.6*	46.8 ± 1.4*	45.0 ± 1.1*	56.3 ± 0.7*

two gear types. Catch statistics for each trip calculated using the imposed cull sizes of 70, 80, and 90 mm shell are shown in Table 4.

During August and September of 1997, the sea scallop resource consisted of few age 3 sea scallops (70–90 mm sea scallops) and relatively low numbers of age 3+ (>90 mm)sea scallops. For these two trips, the total number of sea scallops caught per hectare was not significantly different ($P > 0.05$) at the 70 and 80 mm shell height cull sizes. When the cull size was increased to 90 mm, the dredge vessels captured significantly more ($P < 0.05$) sea scallops per hectare than did the trawl vessels. The dredge vessels were able to produce significantly more ($P < 0.05$) scallop meats at all culling sizes. These results reflected the differing relative harvest efficiencies and sea scallop abundance and size distribution at the time of the two trips.

The trawl vessel captured and produced significantly more ($P < 0.05$) sea scallops and meats than the dredge vessel at the 70 mm cull size in May 1998 due to the presence of large numbers of 70 to 90 mm sea scallops. When the cull size was increased to 90 mm and 70 to 90 (age 3) sea scallops were excluded from the analysis, dredge vessels captured and produced significantly more ($P < 0.05$) sea scallops and scallop meats relative to than the trawl vessels. Meat counts from the trawl vessels were significantly higher ($P < 0.05$) than meat counts from the dredge vessels for all trips at all culling sizes.

DISCUSSION

The sea scallop resource is in a constant state of flux as a result of variable recruitment, rapidly growing individuals, and high rates of fishing mortality. Sea scallop abundance and size distribution can change dramatically, even during the time scale of this study (August 1997 to May 1998). Despite the changing resource conditions, two general patterns were observed during the three trips. The two resource conditions differed with respect to the presence or absence of an age 3 (70–90 mm shell height) recruiting year class of sea scallops.

Sea scallops recruit to the fishery at 3 years of age. Three year old sea scallops, which in the mid-Atlantic region have a shell height of roughly 70 to 90 mm, represent an important age class in the fishery. As sea scallops grow to 70 to 75 mm shell height, they begin to be retained by commercial vessels (DuPaul and Kirkley 1995, DuPaul et al. 1995). Recent high levels of fishing mortality have reduced the abundance of older sea scallops in the population, and 3-year-old sea scallops that recruit to the gear each year have primarily supported the fishery (Serchuk et al. 1979, NEFMC 1993).

TABLE 4.

Mean number of sea scallops harvested, mean grams of scallop meats produced, and average meats per pound (MPP) for all comparative gear trips. Values are calculated using assumed culling sizes of 70, 80, and 90 mm shell heights, standardized to reflect catch per hectare covered by the gear.

	Trip 1 (August 1997)		Trip 2 (September 1997)		Trip 3 (May 1998)	
	Stephanie B. Dredge (n = 34)	Triangle 1 Trawl (n = 77)	C. Breeze Dredge (n = 30)	Capt. AT Trawl (n = 49)	C. Clipper Dredge (n = 29)	Triangle 1 Trawl (n = 14)
Harvest (#/ha.)						
Cull at 70 mm	71.7 ± 2.5	70.0 ± 4.0	61.5 ± 2.9	60.6 ± 5.6	110.1 ± 9.7*	264.7 ± 50.5*
Cull at 80 mm	67.9 ± 2.3	59.2 ± 3.4	58.4 ± 2.7	49.6 ± 5.1	67.3 ± 3.4	71.3 ± 6.8
Cull at 90 mm	46.6 ± 1.5*	26.5 ± 1.7*	43.2 ± 2.3*	20.9 ± 2.2*	40.0 ± 2.1*	10.3 ± 1.0*
Production (grams/ha.)						
Cull at 70 mm	1,088.6 ± 34.3*	816.2 ± 44.5*	918.1 ± 45.4*	688.7 ± 62.1*	1,399.5 ± 84.8	2,111.1 ± 354.7
Cull at 80 mm	1,062.0 ± 33.3*	743.4 ± 41.4*	897.6 ± 44.88*	611.7 ± 60.0*	1,096.5 ± 53.3*	768.6 ± 70.7*
Cull at 90 mm	834.3 ± 28.6*	397.0 ± 24.9*	734.2 ± 43.3*	311.7 ± 32.5*	824.0 ± 45.1*	170.3 ± 15.4*
MPP						
Cull at 70 mm	36.7 ± 0.4*	46.0 ± 0.7*	36.9 ± 0.7*	46.4 ± 1.3*	47.4 ± 1.2*	63.7 ± 1.1*
Cull at 80 mm	34.9 ± 0.4*	41.3 ± 0.3*	35.1 ± 0.6*	41.0 ± 0.9*	35.6 ± 0.5*	48.2 ± 0.2*
Cull at 90 mm	30.0 ± 0.3*	34.0 ± 0.1*	31.3 ± 0.4*	33.6 ± 0.4*	25.8 ± 0.4*	31.3 ± 0.7*

Shell height distributions for trips 1 and 2 portray a population that was characterized by a low abundance of age 3 sea scallops. The absence of large numbers of 3-year-old sea scallops had a large impact on the relative production rates of the two regulated gear types. The reduced ability of the trawl to capture sea scallops greater than 90 mm relative to the dredge, coupled with a minimum observed crew cull size of roughly 70 to 75 mm resulted in trawl boats being dependent upon 3-year-old sea scallops for production. In the absence of large numbers of age 3 sea scallops, production rates of the dredge vessels in terms of numbers of sea scallops captured per unit area and weight of scallop meats produced exceeded those from the trawl vessels during the first two trips.

During August 1997 (trip 1), large numbers of 40 to 60 mm shell height sea scallops were observed in the catches of both the dredge and the trawl. Growth of this cohort over the next 9 months resulted in these sea scallops attaining a shell height range whereby they were recruiting into the fishery the following spring. During the May 1998 trip, age 3 sea scallops from this cohort were captured in numbers 5 to 6 times greater than the previous trips in 1997. The presence of this strong age 3 year class had a profound effect on the relative production rates of the dredge and trawl vessels. When age 3 sea scallops were present in large numbers, the trawl vessels catch per hectare was 35.4% greater than that of the dredge vessel. The observed shift in relative harvest efficiency and the resulting ramifications in relation to production rates demonstrated an inherent inequality between the two regulated gear types.

Irrespective of changing resource conditions, a significant shift in relative harvest efficiency at 90 to 95 mm shell height was observed over all three trips. Trawl vessels were more efficient at capturing sea scallops less than 90-mm shell height relative to the dredge vessels. At shell heights greater than 90 mm, the trawl vessels were observed to operate less efficiently relative to dredge vessels. This shift in relative harvest efficiency had a large effect on catch compositions and ultimately production rates. The magnitude of the observed differences were dependent on the resource conditions at the time and location of the study. DuPaul et al. (1989c) observed similar results in comparing pre-Amendment 4 scallop trawls and dredges. At approximately 90 mm shell height, the 3 inch (76 mm) ring dredge started to perform more efficiently relative to the trawl nets used in the study.

The shift in relative harvest efficiency may be explained by behavioral characteristics of the sea scallop. Sea scallops less than 100 mm shell height have been found to be highly mobile (Caddy 1968, Dadswell and Weihs 1990), and have been observed to elicit a flight response at the approach of a dredge (Caddy 1968, Worms and Latienge, 1986). As scallops grow larger than 100 mm, mobility decreases and these larger animals become sedentary, living in shallow depressions created in the substrate (Bourne 1964). A dredge which is designed to scrape the substrate may be able to capture larger sea scallops (>100 mm shell height) found in slight depressions in the substrate. A trawl that skims over the substrate may not be able to capture these larger sea scallops as efficiently as the dredge.

Size Selectivity

Gear selectivity occurs as a scallop enters a trawl or dredge on the sea floor. Selection properties of the gear dictate whether a scallop escapes or is captured, and is primarily a function of scallop size relative to the mesh or ring size in the trawl or dredge. Sea scallops that are too small to be retained by the gear pass through spaces in the meshes, rings, or inter-ring spaces. Selection by the crew occurs when the catch is dumped on deck and the crew culls the catch for sea scallops to be retained for shucking. Under Amendment 4, no meat count restrictions exist and it is up to the discretion of the captain and crew to establish the size of sea scallops that are retained for shucking.

Traditional size selectivity studies are based on a comparison between length frequency distributions from an experimental (selective) versus a control (non-selective) gear. The non-selective gear provides an estimate of the size distribution of the animals that pass through the meshes or rings of the experimental gear. Covered codends, small mesh codends, and small mesh liners represent some non-selective devices utilized in the literature (Hodder and May 1965, Pope et al. 1975, Serchuk and Smolowitz 1980, DuPaul et al. 1989a, Wileman et al. 1996). The length frequency distribution from the non-selective gear is then compared with the catch from the experimental gear to generate a size selection curve.

A non-selective gear was not used to determine absolute selectivity in this study. The data collected represented the catch from two experimental (selective) gear configurations. With no estimate of the length frequency distribution of sea scallops that passed through the rings of the dredge and meshes of the trawl, absolute selection curves could not be generated. Millar (1995) states that comparative gear selectivity experiments in which no control is used can not provide conclusive evidence of any selection curve because any fit to the data can arise from an infinity of selection curve models. In the absence of an estimate of absolute gear selectivity, relative gear selectivity can be inferred from length frequency distributions, catch compositions, and relative efficiency estimates.

Results of the crew size selectivity analysis suggest a standard for minimum retention size. DuPaul and Kirkley (1995) reported that sea scallops begin to be retained by the fishery at roughly 70 to 75 mm shell height. Our findings corroborate this observation, as the L_{50} values over all trips ranged from 69.3 to 77.5 mm. DuPaul et al. (1995) and DuPaul and Kirkley (1995) observed that crew culling practices changed in response to a dominant year class that grew over the course of the study period. In this study, however, no shift in sea scallop size selection was observed even though the size composition of the catch varied widely over the three trips.

Implications for the Fishery and Management

Controlling age at entry is one management strategy used to maximize yield per recruit and increase the spawning potential of the managed population. Serchuk et al. (1979) estimated that maximum yield per recruit for sea scallops is attained at an age of first capture of 8 years. Only minor increases are realized as age at first capture increases from ages 6 to 8. While it may be unrealistic to delay the age at first capture to 8 or even 6 year old sea scallops, significant benefits in terms of yield per recruit can be realized if sea scallops are allowed to reach age 4 before recruiting to the fishery. Serchuk et al. (1979) estimated an increase of 39% in yield per recruit for mid-Atlantic sea scallops if harvested at 97 mm as opposed to 77 mm shell height. Similarly, Caddy (1972) estimated a 65% increase in yield per recruit if sea scallops were allowed to grow from 73 to 92 mm shell height. The harvest of 3-year-old sea scallops compromises the management objective of maximizing yield per recruit.

In addition to increasing yield per recruit, delaying age at first capture from age 3 to 4 also adds to reproductive potential in terms of egg production. Age 3 sea scallops produce from 10 to 13.5 million eggs, while 4-year-old sea scallops will produce as many as 22 to 34 million eggs (MacDonald and Thompson 1985, Langton et al. 1987). While exact fecundity estimates vary, age 4 sea scallops can produce 2 to 3 times more eggs than age 3 sea scallops. McGarvey et al. (1993) found a statistically significant spawner-recruit relationship for sea scallops on Georges Bank, and determined that age 3 and to some extent age 4 sea scallops did not measurably contribute to egg production and recruitment on Georges Bank. The harvest of age 3 sea scallops may at best represent a large reduction in spawning potential or possibly the removal of animals before they have had a chance to reproductively contribute to the population.

Equity

The examination of equity between different regulated gear types found in Amendment 4 was an objective of this study and was predicated on relative size selectivity and efficiency. Analyses of shell height frequencies, catch compositions, and relative harvest efficiency indicated that regulated trawls and dredges appear quite different in relation to both size selectivity and harvest efficiency.

Future attempts at equating dredges and trawls in relation to size selectivity could be accomplished through comparative gear research. Studies utilizing differing diamond or square mesh sizes would result in the escape of greater numbers of pre-recruit (<70 mm shell height). Previous comparative gear studies demonstrated that modifications such as increasing ring and mesh sizes reduced, but did not eliminate, the capture of smaller sea scallops, and often reduced overall harvest efficiency (DuPaul et al. 1989c, DuPaul and Kirkley 1995).

While size selection properties of sea scallop gear seem to be broad, the crew culling process has been shown to be very selective. Assuming the majority of sea scallops that are discarded survive the capture and culling process, the crew culling process in combination with more selective gear types has the potential to be an effective tool in controlling scallop size at entry into the fishery process (Medcof and Bourne 1964, DuPaul et al. 1995, DuPaul and Kirkley 1995).

Sea scallop trawls were observed to have a reduced ability to capture sea scallops greater than 90 mm relative to standard sea scallop dredges. This differential harvest pattern coupled with an observed minimum culling size at 70 to 75 mm implies that trawl vessels will depend, in a large part, on age 3 sea scallops for landings. If the resource consists of large numbers of sea scallops less than 90-mm shell height, dredge vessels will be at a competitive disadvantage relative to trawl vessels. Management strategies have clearly pointed to the objective of restoring the abundance and age distribution of the adult stocks (NEFMC 1982). If resource composition is restored in the future, sea scallops greater than 90 mm will represent a larger proportion of the resource. The ability of dredge vessels to more efficiently harvest sea scallops larger than 90 mm shell height dredge vessels will result in a competitive advantage for dredge vessels relative to trawl vessels. This generalization is dependent upon the relative abundance of scallop size classes present in the population.

The reduced ability of trawls to capture sea scallops greater than 90 mm shell height relative to the dredge may make equating the two gears difficult. Future trawl design modifications may be able to reduce the catch of small sea scallops, but results from this and previous studies suggest that current trawl designs may not be able to harvest larger sea scallops as efficiently as scallop dredges (DuPaul et al. 1989c). Once trawl and dredge designs are engineered to have similar selectivity patterns, the issue of harvest efficiency could be addressed. Harvest efficiency is partly a function of gear width, or the area over the bottom that the gear can cover. Currently, gear width is mandated to be a maximum of 30 ft. (9.0 m) of dredge width and 144 ft. (43.2 m) of trawl sweep. Modifications of gear width could possibly equilibrate the two gears in relation to relative harvest efficiency.

The comparison of relative efficiency and size selectivity of the two regulated gear types represents the first comparative level of analysis on how dredge and trawl vessels operate. To adequately compare the two gears, a broader view of how dredge and trawl vessels operate at the fleet level should be examined. Trawl vessels hold 22% of the total permits in the fishery and account for 10% to 15% of the annual landings. Trawl landings for the 1998 to 1999 fishing year were 1.29 million pounds, or 11% of the total landings (NEFMC 1999). Trawl vessels tend to operate out of ports in the mid-Atlantic region and are operationally limited to working in areas of smooth, clean bottom. As a result of this limitation, trawl vessels can operate in only a fraction of the area that is available to the dredge boats. Therefore, only a limited portion of the scallop resource is subject to harvest by sea scallop trawl gear. Intense fishing activity by trawl vessels in this limited resource area may result in dramatic localized effects to incoming year classes of 70 to 90 mm shell height sea scallops.

This study demonstrated that the assumptions that formed the basis of the gear regulations found in Amendment 4 were not entirely correct. Clearly, if a management objective is to require that sea scallop trawls and dredges have equivalent size selection and relative efficiency, more comparative gear research is a necessity. In general, quantifying the role that different fishing gears have on the utilization of the sea scallop resource is an objective yet to be fully achieved.

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A SHIPMENT METHOD FOR SCALLOP SEED

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ABSTRACT A "sandwich" made of layers of sponge and plywood lids placed inside plastic bags and packed in styrofoam coolers was evaluated as a device for shipment of catarina scallop (*Argopecten ventricosus* Sowerby II, 1842) spat. Spat survival was measured to evaluate the combined effect of temperature, emersion time, sponge thickness, spat density, shell gapping, and O_2 levels. In addition, temperature inside the coolers with different amounts of ice was measured under different external temperatures. Results indicate that scallop seed can successfully be shipped out of the water over long periods of time (>37 h), taking advantage of the scallop's capacity to breathe in air. Survival was greatly enhanced when a pure-O_2 atmosphere was used. Highest survival was obtained at lower temperature and at shorter emersion times. Survival was not affected by either sponge thickness or by spat density. Higher rates of survival occurred when shells were forced to remain tightly closed. Mortality was attributed to a combination of O_2 shortage and desiccation, but the possibility of a genotoxic effect of several metabolites and toxicity by nitrogenous compounds as possible causes are discussed. To maintain temperatures in the coolers, 50 g L^{-1} of ice was found to be optimum.

KEY WORDS: live transport, scallop, emersion, anaerobiosis, *Argopecten ventricosus*

INTRODUCTION

Shipment of live aquatic organisms currently is performed either in tanks with aerated water or out of the water under moist conditions. The first method is appropriate for only short-term shipments (a few hours) because of bacterial growth, the presence of dissolved material voided in the feces (Bayne 1976), O_2 depletion, and the accumulation of toxic nitrogenous excretion products such as ammonia, which quickly reduce the quality of the water in which the animals are being transported. In addition, this method is expensive because of the cost of shipping water together with the animals. Conversely, shipment without water under moist conditions is comparatively inexpensive but exerts a physiological cost to the animals, which results in high mortality if shipping conditions are not appropriate.

Rhodes and Manzi (1988) wrote about a shipping method for clam and scallop seed at different stages for different emersion times for up to 6 days. The only information given was that there was a direct correlation between shipping duration and mortality of bivalve seed. The greatest mortality occurred in the smallest size bivalves shipped over the longest period of time. The highest survivals were for the largest size bivalves shipped over the shortest period. Hard clams had greater overall survival than scallops at almost all size classes and shipping durations. No further information on temperature, emersion time, or survival was given.

To find an adequate out-of-water shipping method for scallops, background information is needed. Contrary to the majority of bivalves, scallops are monomiarian bivalves that gap their shells when exposed to air, causing desiccation. *Pecten maximus*, as all scallops, initially responds to air exposure by violent adductions of the shell and tachycardia, followed by gradual bradycardia, accompanied by wide gapping of valves. Inability to control air gapping and consequent vulnerability to desiccation is a characteristic feature of scallops similar to other sublittoral species (Brand and Roberts 1973). Desiccation could be avoided in transport by forcing the scallops to keep their shells closed while in a highly moist atmosphere, although loss of water may occur through the bysal notch of the shell.

If desiccation can be prevented or reduced, how the animal is going to respire during shipment should be considered. Anaerobic respiration is a common alternative used by many intertidal molluscs in the events of anoxia or exposure to air (Shumway and Scott 1983, Devi et al. 1984, Maeda-Martínez 1987, Aunaas et al. 1988, Marshall and McQuaid 1989, Vial et al. 1992, Oeschger and Storey 1993, Wang and Widdows 1993, de Zwaan et al. 1995, Simpfendörfer et al.1995). However, scallops do not seem to be adapted to glycolysis pathways for this purpose (de Zwaan et al. 1980, Thompson et al. 1980). The remaining alternative for the scallop to breathe during shipment is to gain oxygen from the atmosphere, to which the respiratory apparatus is ill adapted. The air breathing capacity in scallops has not been demonstrated. However in other bivalves such as *Mytillus californianus*, *Modiolus demissus*, and *Cardium edule*, air breathing was found to be 63%–74% of the standard rate of oxygen consumption in water at the same temperatures (Kuenzler 1961, Boyden 1972a, Bayne et al. 1975). The median survial time of *M. demissus* in air is proportional to the amount of oxygen present (Lent 1968), and therefore an atmosphere of pure oxygen might enhance survival. The use of pure O_2 for shipping animals has not been reported. Pure O_2 is currently in use in fish and shrimp aquaculture industries to supersaturate the water in which the animals are transported.

Temperature is considered the most important factor determining the level of activity in poikilotherms (Bayne 1976). Therefore, within limits, a decrease in ambient temperature may improve survival during shipment because the amount of oxygen required (which is limited inside the shipment device) for the respiration of the animals under transport will be reduced. Therefore, optimum temperature for shipment is critical. Optimum temperature for growth in *Argopecten ventricosus* is 19–22 °C (Sicard et al. 1999)

but the median lower lethal temperature is not known. From the literature, it is known that the species may withstand temperatures as low as 12 °C because they have been captured in the continental shelf of the Baja California Peninsula at a depth of 180 m, where a temperature of 12 °C has been measured (Maeda-Martínez et al. 1993). At this temperature the respiration rate (VO_2) was 0.5–1.0 mL O_2 g^{-1} h^{-1}, whereas at 28 °C , VO_2 was three times higher (3.1 mL O_2 g^{-1} h^{-1}) (Sicard et al. 1999).

If the optimum temperature range for shipment of the species is known, the problem is to produce and maintain the temperature within that range during the trip. The current method for shipping live animals uses styrofoam coolers of different shapes and thicknesses and employs frozen bricks of blue ice to lower the temperature. The insulation capacity of the cooler can be provided by the manufacturer, but this is of little use when the amount of ice bricks are not standardized and large variations in external ambient temperature occur. In the tropics, a cooler may be exposed to temperatures from freezing (if the boxes are placed in the nonthermoregulated compartment of the plane during flight), to 40 °C or higher if exposed to direct sunlight. Because of this, it is important to determine the temperature variations inside a styrofoam cooler containing a known amount of ice and exposed to different ambient conditions.

We have developed a shipping device and evaluated it for shipping scallop seed. Scallop survival was measured at different temperatures, emersion periods, spat densities and shell gapping levels. The effect of sponge thickness and the effect of a pure-O_2 atmosphere against a normal air atmosphere on scallop survival were estimated. The cooling effect of different quantities of blue ice was measured inside styrofoam coolers exposed to sunlight and under shade.

MATERIALS AND METHODS

Experimental Animals

Fourteen thousand catarina scallop juveniles (3.5 ± 0.4 mm shell-height and 3.7 mg dry tissue weight) produced at the hatchery of CIBNOR La Paz, Mexico were used in the study.

Shipping Device

The shipping device was made with three layers of sponge rubber (20 × 10 cm) placed between two 1/8-inch plywood rectangles of the same size (Fig. 1). The scallop seed was placed on the first (bottom) and second (middle) layers. The top layer served as a lid. The sponges were soaked with seawater before use. A plastic tie was used to secure the sandwich. The sandwiches were placed inside 2-L plastic bags, filled with air or pure O_2 before they were closed and sealed with a rubber band. The approximate gas volume inside the bags was 600 mL . The 7-cm high sandwich had a volume of 1.4 L (20 × 10 × 7 cm).

Experimental Design

Spat survival was measured to evaluate the combined effect of three different temperatures (17, 22, and 28 °C), three emersion times (19, 26, and 37 h), two sponge thicknesses (1.25 and 2.5 cm), two spat densities (2 and 25 scallops/ cm^2), two shell gapping levels (tight and loose), and two O_2 atmospheric conditions (air-only and pure-O_2). In the pure-O_2 experiments, only thick sponges were tested. Each treatment was made with one replicate. The spat

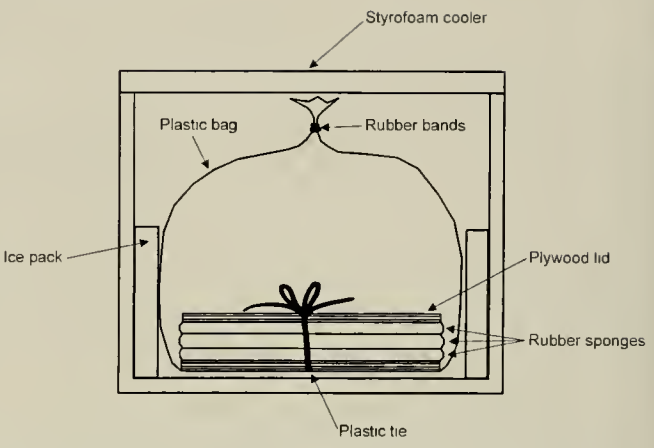

Figure 1. Device used for shipping catarina scallop (*Argopecten ventricosus*) spat.

from each treatment were removed at the emersion times and were placed in 1-L glass containers with seawater at 37‰ and at the experimental temperatures to allow the scallops to recover from exposure to air. Each container received constant aeration and 150,000 cells/mL of a mixture of cultured microalgae (*Isochrysis galbana* and *Chaetoceros gracillis*). The spat remained at least 2 h in these containers before the dead and the live animals were counted.

The experimental temperatures were achieved by placing the sandwiches in temperature-controlled rooms at 17, 22, and 28 °C. Shell gapping (tight and loose) was produced by the strength at which the sandwiches were secured. In the former, the sponge maintained a moist atmosphere around the animals but care was taken not to compress the animals. In the latter, the shells were forced to remain tightly closed, securing the sandwich as tightly as possible. To test the density (spat cm^{-2}) effect on survival, each sponge was marked at the middle with a pen marker. About 100 seed were spread over one-half of the sponge at a density of two scallops cm^{-2}, while in the other half the seed was heaped up covering only 4 cm^2 at 25 scallops cm^{-2}. Each sandwich then held 400 spat.

The pure-O_2 experiments were done replacing the air in the plastic bags with medicinal O_2 from a cylinder. Each bag holds approximately 2 L of gas.

Styrofoam Cooler Temperatures

The temperature changes inside 27.3-L styrofoam coolers (39 cm long × 28 cm wide × 25 cm deep and 2.2 cm thickness) containing 4 or 8 0.35-kg blue ice bricks (50 or 100 g of blue ice per liter of cooler) were monitored over 48 h with a data logger set to record temperature every 0.5 h. This allowed continuous readings with 0.01 °C precision. The coolers were similar to those used commercially for shipping live animals, such as shrimp postlarvae. One set of coolers was exposed to direct sunlight, another was placed in the shade, and a third was kept in a temperature-controlled room at 22 °C. The temperature fluctuations in the coolers of the last treatment were only tested using eight blue-ice bricks. These results were contrasted against ambient temperature in the shade, which was simultaneously recorded by the meteorological station at CIBNOR.

RESULTS

Air-only Experiments

In the air-only experiments, survival was higher at shorter emersion times and at lower temperatures. The highest survival was 61% at 17 °C, using thick sponge, at low density, with tight shell gapping, and at the shortest emersion time tested (19 h). Negligible survival was obtained after 26 h in all air-only treatments. A Tukey multiple-range test indicated significant differences between survival at 17 °C-19 h and at warmer temperatures and longer emersion times.

To evaluate differences among sponge thickness, spat density, and shell gapping treatments, Tukey multiple-range tests were made. Results show that survival was not affected either by sponge thickness or by spat density. However, a significant difference between shell gapping levels was found at $P > 0.01$. Higher survival was obtained when shells were forced to remain tightly closed.

Pure-O_2 Experiments

Figure 2 shows the comparative results of pure-O_2 experiments against their corresponding air-only treatments. From this, survival in pure-O_2 was significantly higher than their corresponding air-only treatment, which confirms the scallop capacity for air respiration and the advantage of using a enriched O_2 atmosphere. Average survival in pure-O_2 experiments under the most adverse conditions of highest temperature (28 °C) and longest emersion time (37 h) was 20%. At 17 °C and 19 h of emersion, average survival was 97%. A significant effect of shell gapping was obtained here as in the previous air-only experiments at $P > 0.01$. Survival was greater when the shells of the scallops were tightly closed.

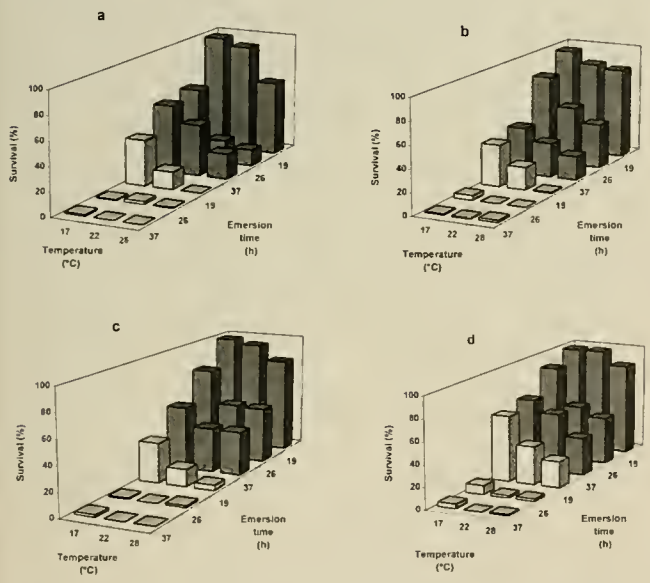

Figure 2. Survival of catarina scallop (*Argopecten ventricosus*) spat (3.5 mm shell height; 3.7 mg dry tissue weight) at different temperatures and emersion times, and incubated in the shipment device under a pure-O_2 (gray columns) and air-only (white columns) atmospheres. a = high density-loose, b = high density-tight, c = low density-loose, and d = low density-tight.

Styrofoam Cooler Temperatures

Variations in ambient shade temperatures and inside styrofoam coolers with 50 and 100 g L^{-1} of ice exposed to direct sunlight, in the shade, and in a temperature controlled room at 22 °C for 48 h are shown in Figure 3. Average ambient temperature was 29.4 °C with a maximum of 38.2 °C and a minimum of 21.4 °C. Independently of the quantity of ice employed, temperature inside the coolers dropped from ambient temperature (≈25 °C) to the lowest value of 5.9 and 14.5 °C in the 100 and 50 g L^{-} treatments within the first 4 h of the experiments. Using 100 g L^{-1} of blue ice, a temperature shock of 5 °C h^{-1} was produced, which could probably be lethal to scallops. In addition, the low temperature reached (5.9 °C) may exceed the lower thermal limit of the species. With 50 g L^{-1} of blue ice, temperature also fell in 4 h, but the lowest temperature was only 14.5 °C, which is higher than the lowest temperature (12 °C) at which the catarina scallop has been captured (Maeda-Martínez et al.1993). Once the lowest temperature was reached, it began to increase steadily (Fig. 3), and the velocity of the increase was a function of the quantity of ice employed and of the external conditions. In the 50 g L^{-1} treatment, temperature increased to about the average external ambient temperature of 29.4 °C (Fig. 3a) 25 h from the start of the experiments, whereas in the 100 g L^{-1} treatments, temperature never reached equilibrium with the external medium in the 48 h of the experiment (Fig. 3b).

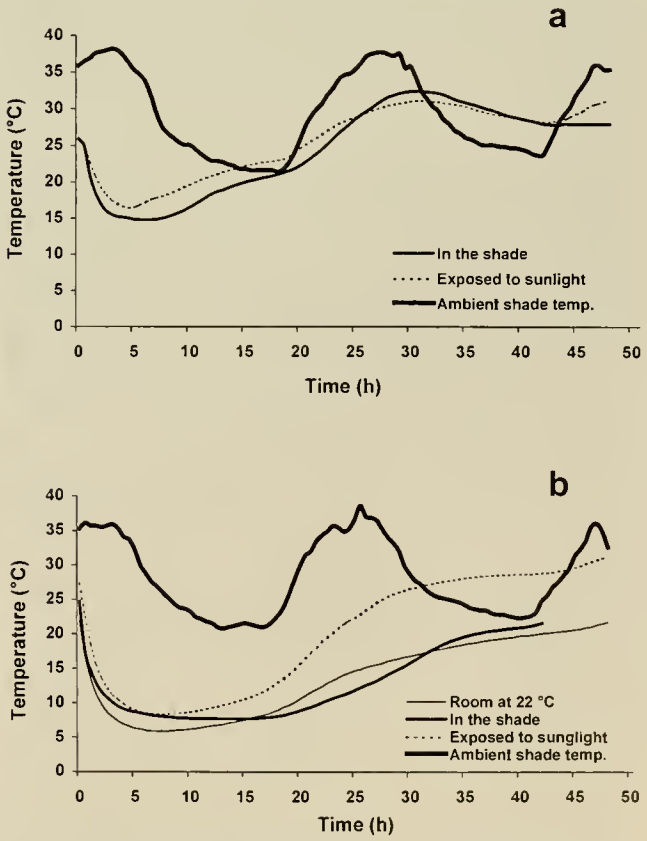

Figure 3. Temperature variations inside 27.3-L styrofoam coolers containing 4 (a) and 8 (b) blue ice bricks, each weighing 0.35 kg, over 48 h. Coolers were exposed to direct sunlight, kept in the shade, and in a temperature controlled room at 22 °C. This figure also shows the ambient temperature in the shade.

To determine the temperature conditions that would be expected at the same emersion times as the previous experiments, the extreme temperatures were obtained from the data logger at 19, 26, and 37 h (Table 1). In the 100 g L^{-1} treatment, extreme temperatures remained the same at the different emersion times regardless of the external variations in temperature (Fig. 3b). The cooling effect of the blue ice bricks lasted for more than 19 h. In the 50 g L^{-1} treatment, the maximum temperature exceeded the median lethal temperature (29 °C) of the species (Sicard et al. 1999) 26 h from the beginning of the experiment under both external conditions tested (Table 1).

DISCUSSION

The results indicate that scallop seed can be successfully shipped out of water over long periods of time (>37 h), taking advantage of the scallop's capacity to breathe in air. This capacity was not expected because many invertebrates exposed to air show different responses because of their different behavioral strategies and physiological tolerances. These tolerances are certainly greater in intertidal than in subtidal species, because intertidal species might experience short-term exposure to air on a daily basis at low tide. Subtidal species, such as *A. ventricosus*, are rarely exposed in their natural environment.

It may seem odd that many intertidal invertebrates enter anaerobiosis at low tide when there is access to atmospheric oxygen. But for essentially all aquatic invertebrates, the desiccation stress when exposed to air may be so severe that many species isolate themselves within closed shells. At low tide the facultative anaerobe *Mytilus* must cease gill irrigation and remain with its valves tightly closed, shifiting to anaerobiosis (Bayne 1976). As an advantage, *Mytilus* does not produce lactic acid as an end product but the less toxic alanine and succinate following a modified glycolytic scheme (Wells 1980). Although anarobiosis has been demonstrated to play an important role in intertidal mollusks during exposure to air, it is unlikely that this pathway would be used by the scallop. Anaerobic respiration in scallops is predominantly used for energy production during sudden bursts of activity such as swimming or the valve-snapping escape response (de Zwaan et al. 1980, Thompson et al. 1980). Phosphoarginine is used as the main energy source and octopine is produced as an end product (Bricelj and Shumway 1991). The scallop adductor muscle, which is the main storage organ, only contains 18 to 25 % of glycogen, whereas in *Mytillus edulis*, an intertidal bivalve that commonly uses anaerobic pathways during prolonged valve closure, attains high maximum seasonal glycogen levels of 42 to 53% in the mantle, the principal long term storage organ in mytilids (de Zwaan and Zandee 1972, Gabbott 1983).

On air exposure, Lent (1968, 1969) and Boyden (1972a) reported that groups of *Modiolus demissus* (33%) and *Cardium* spp. (42%) tolerated those loses of weight as water before mortality occurred. We did not measure water loss but direct evidence of this was the significant difference found between survival of tight and loose shell-gapping treatments. The shipping method described minimized desiccation by forcing the shells of the juveniles to remain closed. Water may have been lost through the bysal notch of the shells. Although the rubber sponge helped to maintain a moist environment around the seed, this does not seem to be an optimum material because the upper layers of the sandwich were dryer than the bottom layers at the end of the experiments. This explains the lower survival observed (not quantified) from upper layers than from bottom layers in all treatments. If this is so, the method can be optimized either with the use of a spongy material with higher hydrophilic properties or by placing a layer of absorbent paper between the seed and the sponge.

No differences in spat survival were found at different densities. This indicates that the seed could be piled up during shipment, which will reduce the number of shipping containers needed and costs. However, this does not indicate the number of scallops that can be shipped per unit of bag volume. In both treatments (2 and 25 spat cm^{-2}), the same volumetric density of 400 spat /600 mL was tested, assuming that only 30% of the bag volume was occupied by gas (2.0 L bag-1.4 L sandwich). The volumetric density used was 0.66 spat mL^{-1} or 2.4 mg dry tissue weight (dtw) mL^{-1}, if the dtw of a 3.5 mm shell-height spat was 3.7 mg. From this, a total of 5,600-3.5 mm spat or 20.2 g dtw can be shipped successfully with results similar to our experiments, using a 28-L commercial styrofoam cooler. These numbers and biomass per cooler are low and probably not economically useful. Further research is needed to find the optimum volume density for shipment.

The differences in survival between air-only vs. pure-O_2 experiments may suggest that mortality in the former was because of a shortage of oxygen. In *Modiolus demissus*, the median survival time in air is proportional to the amount of oxygen present (Lent 1968). Boyden (1972b) showed that by preventing *Cardium edule* from gapping, its survival in air was significantly reduced. However a straight forward explanation like this cannot be given for the mortality in the pure-O_2 treatments because survival from some air-only and pure-O_2 experiments were similar, as in 17 °C-19 h air-only and 17 °C-37 h pure-O_2 low density-tight treatments (Fig. 2d), though a much higher oxygen content in the pure-O_2 bags would be expected than in the air-only experiment. To test this hypothesis, final O_2 available in the bags at the different treatments has to be determined. For this, the oxygen consumption of the spat has first to be estimated. Unfortunately the O_2 consumption in air was not measured during the experiments, but a good approach could be made if the O_2 uptake in air was assumed to be about 70% the standard respiration rates ($VO_{2,s}$) in water determined by Sicard et al. (1999) in this species, as in other bivalves (Kuenzler, 1961, Boyden, 1972a, Bayne et al. 1975). In the catarina scallop, the relationship between $VO_{2,s}$ and temperature is described by the equation ($n = 6$; $r = 0.98$):

$$VO_{2,s} = 0.047\, e^{0.1413\, T°C}$$

From this, the hypothetical oxygen concentrations at the end of

TABLE 1.

Maximum and minimum temperatures (°C) recorded inside 27.3-L styrofoam coolers contaioing 1.4 and 2.8 kg of blue ice (50 and 100 g L^{-1})

	19 h (n = 38)		26 h (n = 52)		37 h (n = 74)	
Experimental conditioos	Max	Mio	Max	Min	Max	Min
50 g L^{-1} in the shade	25.9	14.8	29.4	14.8	31.2	14.8
50 g L^{-1} exposed to sunlight	26.1	16.5	29.7	16.5	32.5	16.5
100 g L^{-1} in a room at 22 °C	22.0	5.9	22.0	5.9	22.0	5.9
100 g L^{-1} in the shade	24.7	7.7	24.7	7.7	24.7	7.7
100 g L^{-1} exposed to sunlight	27.3	8.4	27.3	8.3	28.5	8.3

Coolers were exposed to direct sunlight, in the shade, and in a temperature-controlled room at 22 °C at different incubation times.

the air-only and the pure O_2 experiments at different temperatures and emersion times, were calculated (Table 2). In this table, the total amount of oxygen consumed (TVO_2) on emersion was obtained with the formula:

$$TVO_2 = (VO_{2\ s})(0.7)(dtw)(t)$$

where dtw was the total biomass incubated in the bags (400 spat × 3.7 mg dtw = 1.48 g) and t was the emersion time. For the estimation of TVO_2, it was assumed that VO_2 varied independently from the available oxygen (PO_2). In the catarina scallop, VO_2 has been found to remain independent of PO_2 only in the range between 100 and 76% O_2 saturation (Sicard et al. 1999). At lower oxygen concentrations, VO_2 became dependent on PO_2 and therefore TVO_2 overestimated the oxygen consumption in the experiments, reflecting the maximum O_2 uptake possible by the scallops during emersion. With these results, we conclude that in pure-O_2 experiments there was sufficient oxygen and that mortality was produced by a combination of other factors.

The other factors that may have contributed to the scallop's mortality besides shortage of O_2 and desiccation, could have been the genotoxic effect of emersion (Brunetti et al. 1992) and to a minor degree the accumulation of toxic nitrogenous compounds. Brunetti et al. (1992) found that on emersion and exposure to anoxic seawater, the frequency of micronuclei in gill tissues of *Mytilus galloprovinciallis* rose as a function of time. They suggested that the genotoxic agent may be a product of anaerobic metabolism such as propionic or acetic acid. For the toxicity by nitrogen compounds, Bayne et al. (1975) found that the rate of production of ammonia in *Mytillus californianus* was only about 5% of the immersed rate during exposure to air.

As expected, our results showed that spat survival was higher at lower temperatures (17 °C) and at shorter emersion times (19 h). Within limits, metabolism in poikilotherms varies with temperature. As a consequence, O_2 demand diminishes as temperature decreases, increasing the resistance of the animal to prolonged emersion. The problem arises when a desired range of temperature has to be maintained during shipment. We demonstrated that 100 g L^{-1} of blue ice may be effective in keeping the temperature lower than the upper thermal limit of the species (29 °C) for more than 48 h, even at ambient temperatures above 38 °C. However this quantity of ice will probably kill the animals as temperature falls beyond the lower thermal limit of the species. Using half this quantity (50 g L^{-1}), the problem of low temperature can be eliminated because temperature only decreases to 14.5 °C, a temperature within the tolerable thermal range of the species (Sicard et al. 1999). However with 50 g L^{-1} of ice, shipment times cannot last longer than 26 h at ambient temperatures of 38 °C because temperature in the coolers may exceed the upper thermal limit of the species. This problem can be solved by using a thicker cooler with greater insulation capacity. Another problem to be solved is the thermal shock (2.5 and 5.0 °C h^{-1}) given to the animals during the first 4 h of shipment using 50 or 100 g L^{-1} of blue ice. This could be minimized by acclimating the animals to the minimum expected temperature in the coolers and packing them at this temperature in a temperature-controlled room. Further research is needed to determine whether this procedure would improve survival. In *Penaeus japonicus*, 100% survival was obtained in shipments for as long as 17 h out of water by cooling the animals 5 h from 24 °C to 14 °C before air exposure (Samet et al. 1996).

We have devised a successful alternative for shipping scallop spat. The potential application of this method could be the shipment of scallop and other bivalve broodstock, and the transportation of other high-valued species like shrimp, lobster, or abalone to live markets that are currently under expansion throughout the world. The fire hazard that may represent the use of pure oxygen could be reduced, with proper handling and shipping procedures, and by optimizing the amount of pure oxygen used. The optimum O_2-air mixture remains to be determined.

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TABLE 2.

Hypothetical O_2 concentrations in the bags, at the end of the air-only and pure-O_2 catarina scallop (*Argopecten ventricosus*) spat (3.5 mm shell height and 3.7 mg dry tissue weight) shipping experiments.

Temperature (°C)	Emersion time (h)	Standard O_2 uptake rate* in water ($mLO_2^{-1}h^{-1}$)	Total O_2 consumed† on emersion (mLO_2)	Final O_2 air-only‡ (mLO_2)	Final O_2 pure-O_2§ (mLO_2)
17	19	0.5	10.2	115.8	590
17	26	0.5	14.0	112.0	586
17	37	0.5	19.9	106.1	580
22	19	1.1	20.7	105.3	579
22	26	1.1	28.4	97.6	572
22	37	1.1	40.3	85.7	560
28	19	2.4	48.3	77.7	552
28	26	2.4	65.9	60.1	534
28	37	2.4	93.2	32.8	507

Each bag contained 400 spat and 1.47 g dtw total biomass. Figures are assuming an independent O_2 uptake rate from available oxygen concentration.

* Data from Sicard et al. (1999)

† 70% of standard rate in water.

‡ Initial oxygen content = 21% of available bag volume.

§ Initial oxygen content = 100% of available bag volume = 600 mL.

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SEASONAL VARIATIONS IN CONDITION, REPRODUCTIVE ACTIVITY, AND BIOCHEMICAL COMPOSITION OF THE PACIFIC OYSTER, *CRASSOSTREA GIGAS* (THUNBERG), IN SUSPENDED CULTURE IN TWO COASTAL BAYS OF KOREA

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ABSTRACT Seasonal variations in condition index (CI), reproductive activity, and biochemical composition of the oysters, *Crassostrea gigas*, in suspended culture in different nutritional conditions were compared between two bay systems (Jaran Bay and Hansan-Koje Bay) of the southern coast of Korea from January 1996 to September 1997. Differences in temperature and salinity were not significant between stations, but chlorophyll *a* concentrations were significantly higher at Station Josan in Jaran Bay, an outer open system, than at Station Osu in Hansan-Koje Bay, a semi-enclosed bay system with restricted food availability. CI and dry tissue weight of a standard animal showed a similar seasonal cycle, with minimum values in late summer and peaks in spring at both stations. In the Josan oysters a rapid recovery in these components commenced in November 1996 with the simultaneous accumulation of reserves (glycogen and protein) after the summer spawning. By contrast the Osu oysters recovered slowly 3 mo later in February 1997. The values were also considerably higher in Josan oysters than in Osu oysters. At both stations gametogenesis was initiated in late autumn and the breeding period was extended over several months during the summer-early autumn period. Spawning intensity during summer was, however, stronger in the Josan oysters than in the Osu ones. Food availability seemed to be a major factor in determining gonad proliferation and thereby CI, when gametogenesis was initiated. Apparently, the high accumulation of glycogen and protein was observed in the Josan oysters so that the absolute values for the standard animal were significantly higher at Station Josan than at Station Osu. These results indicate that difference in physiological states of the oysters cultivated in the two bay systems are strongly related to site-dependent variation in the storage-utilization cycle of energy reserves (particularly glycogen), depending on food availability. Our findings also suggest that it is necessary to readjust the cultivated density of oysters to procure enough wild seeds and condition of oysters in Hansan-Koje Bay, taking carrying capacity of the bay into consideration.

KEY WORDS: Pacific oyster, *Crassostrea gigas*, condition, reproduction, biochemical composition, suspended-culture, food availability

INTRODUCTION

Seasonal variations in condition and gametogenisis of marine bivalves are strongly related to the energy storage-utilization cycle and environmental factors such as water temperature and food availability (Giese 1969, Gabbott 1975, 1983, Bayne 1976). Bayne (1976) postulated that the cycles of energy storage and gamete production can be overlapped temporally ("opportunistic" species: *Tellina tenuis*, *Abra alba*, and *Cerastoderma edule*) or separated clearly ("conservative" species: *Mytilus edulis*, *Macoma balthica*, and *Pecten maximus*). Recent studies suggested that even within a single species there might be interannual or local differences in the cycles of energy storage and reproduction due to environmental conditions, in particular nutritional condition (Bayne and Worrall 1980, Newell et al. 1982, Rodhouse et al. 1984, Bricelj et al. 1987, Harvey and Vincent 1989, Navarro et al. 1989). These cycles in cultivated bivalves can be different from their wild counterparts (Rodhouse et al. 1984) and among populations of a species from different locations (Brown and Hartwick 1988, Almeida et al. 1997, Okumuş and Stirling 1998). In general, the suspended-culture method of bivalves contributes to high tissue-growth rate due to the better environmental conditions (i.e. food availability; Rodhouse et al. 1984, Pazos et al. 1997).

Traditional culture methods for the Pacific oyster, *Crassostrea gigas*, in the intertidal beds of Korea have been replaced with a suspended-culture method since 1969. Intensive suspended-oyster culture has been developed in the semi-enclosed coastal bays on the south coast of Korea. Owing to the development of oyster culture using ropes suspended from long lines, oyster production in Korea increased abruptly and was maximized up to 288,000 tons in 1987. However, over a recent decade the oyster production was unstable and slowly decreased. Such a recent decrease of oyster production is considered to result largely from local declines of growth rate due to the intensive culture (Yoo et al. 1980) and local shortages in supply of healthy seed oysters (Park et al. 1999).

In some cases of suspended oyster-culturing grounds in the southern coastal bays of Korea, condition of the oysters has been lowered from year to year. Thus the culture period required to produce a marketable product has been prolonged. At the beginning of the development in Hansan-Koje Bay, seeded ropes were suspended in early and mid-summer and then the cultivated oysters were harvested after an approximate 9-mo growth. Recently, this growth period for harvesting is extended to 16 mo, depending on locations. This variability may be explained by the trophic capacity of the bays in relation to the density of cultivated oysters and the availability of food (Héral 1993). Deslous-Paoli and Héral (1988) showed that seasonal variations in the condition and biochemical composition of the cultivated bivalves could be affected by cultivated density at the same area. The densities overstocked within a bay system may also affect the reproductive activity of the oysters. Park et al. (1999) suggested that an overstocked bay, Hansan-Koje Bay (Korea), is unfavorable for the collection of seed oysters and this phenomenon results from extremely low production of larvae due to a prolonged pre-spawning stage and a low prevalence of spawning oysters.

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The high reproductive activity of the oysters is still very important to collect enough seed for the oyster culture of Korea. The condition, which is controlled by both the cycles of energy storage and gametogenesis (Gabott 1975), determines the marketability of commercially exploited bivalve species. Information on the condition and the cycles of gametogenisis and energy storage of oysters in suspended culture is therefore valuable because of biological and commercial interests.

This study investigated the seasonal variation in condition index (CI), reproductive activity, and biochemical composition of suspended-cultivated oysters in two bay systems of the southern coast of Korea. The objectives of the study were to compare physiological states of the oysters cultivated in different environmental (in particular, nutritional) conditions and to examine the site-dependent variation in the role of storage or reserve materials relative to condition and gametogenesis.

MATERIALS AND METHODS

Study Areas

This study was carried out in two bay systems on the southern coast of Korea (Fig. 1). Two locations were chosen for this experiment. One was at Osu in Hansan-Koje Bay with a total area of 56 km^2. This bay is a semi-enclosed system and the cultivated oysters were suspended in the inner site of the bay. The other station was at Josan in Jaran Bay, which is open to oceanic environments. Both of the sites have long been used as farming grounds for the Pacific oyster, *Crassoetrea gigas* with very high cultivated densities compared to other locations.

Oyster Preparation and Biometric Measurements

Oysters (shell length ≈ 7 cm) collected from Koje Bay were cultured on ropes suspended from a long line. Initial density was about 500 individuals per rope. A total of 30 ropes at each station were suspended from 1 m below the water surface and there was a 3-m distance between ropes. Sixty oysters were randomly taken from depth of 1 to 5 m below the water surface at monthly intervals from January 1996 to September 1997. To minimize compositional variations resulting from size class differences, oysters of similar size were sampled. Samples were rapidly transported to the laboratory and placed in filtered seawater at *in situ* temperature for 24 h to evacuate their pseudofaeces and gut contents.

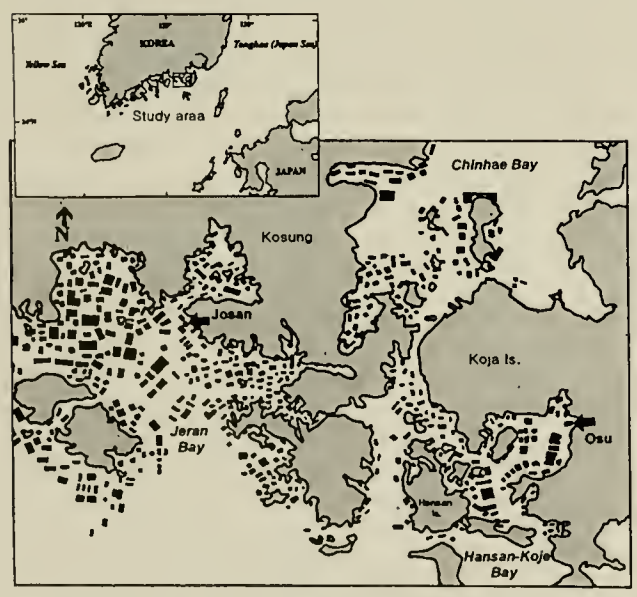

Figure 1. Location of the study area. Black tetragons indicate long-line culturing grounds and arrows represent the sampling stations.

Thirty individuals were cleaned to remove any attached epifauna and adhering sediments, and whole weight was determined for each individual. Shell length, width, and height were measured to the nearest 0.1 mm using vernier calipers. Oysters were then dissected carefully and wet tissue weight was determined after the separated tissues were superficially dried with absorbent tissue paper. The tissues were then frozen and stored at −80 °C until they were analyzed. Shell valves were rinsed with distilled water and weighed after drying in a furnace at 50 °C for 48 h. Dry tissue weight was determined after freeze-drying for 48 h.

Reproductive Activity

Thirty individuals from each sample were used for microscopic examination of histological smears. A transverse cut was made across the body of the oyster and a 3-mm-thick section was fixed in Bouin's solution. It was then routinely processed for histology and 5-μm paraffin-imbedded sections were stained with iron hematoxylin-eosin. The stage of gonadal development was classified and scored on a 0 to 4 scale according to Mann (1979). The arithmetic means of the individual scores of the whole sample was recorded as the Gonadal Maturity Index (GMI) for each sampling date (see details in Dinamani 1987).

Biochemical Measurements

The dry tissue of 30 individuals was pooled and homogenized. The use of pooled tissue from many individuals to determine average biochemical composition may provide useful information because marine invertebrates in the field are often highly variable in biochemical composition (Giese 1967). Ash content was obtained by igniting a subsample (30–80 mg) of homogenized tissue at 450 °C for 48 h in a muffle furnace. Protein was determined by the colorimetric method of Lowry et al. (1951) after extraction with normal sodium hydroxyde. Extraction for total lipid was performed in a mixture of chloroform and methanol (Bligh and Dyer 1959) and lipid content was determined using the method of Marsh and Weinstein (1966). Carbohydrate and glycogen were extracted in 15% trichloroacetic acid and precipitated with 99% ethanol. They were analyzed using the phenol-sulfuric acid method as described by Dubois et al. (1956).

Standard Animal and Condition Index

To present absolute values for biochemical composition, the composition of a standard animal of 22.496 g in dry shell weight was calculated for each sampling date. Allometric equations of $\log_{10}$ dry tissue weight against $\log_{10}$ dry shell weight for each population at each sampling date was determined by linear regression analysis. The results of the biochemical analysis were then expressed in milligrams per standard animal. All regressions were statistically significant ($P < 0.001$) except for June 1996 at Osu station. A similar method was introduced for the clam *Tapes decussatus* L. and *T. philippinarum* by Beninger and Lucas (1984) and for the oyster *C. gigas* by Ruiz et al. (1992). CI was calculated from the dry weights of tissue and shell according to the formula CI = dry tissue weight (mg)/dry shell weight (g) (Walne 1976, Brown and Hartwick 1988).

Environmental Conditions

At each sampling of oyster, water temperature and salinity were measured *in situ* using a CTD meter (Seabird Electronics, Inc.). Duplicate water samples for measurement of suspended particulate matter (SPM) and phytoplankton biomass (chlorophyll *a* concentration) were collected at the water depth of 1 and 5 m with a 3-L van Dorn water sampler. The water was passed through a 250-μm mesh net to remove zooplankton and large particles. Water samples (1–3L) for SPM determination were filtered through a pre-weighed Whatman GF/C glass-fiber filter. The filters were washed with 0.9% ammonium formate, dried at 80 °C, and then reweighed. Chlorophyll *a* concentration was determined on acetone extracts using the fluorometric method as modified by Parsons et al. (1984) with a 10 AU Fluorometer (Turner Designs).

Statistical Treatment

To test the difference between the two stocks of oysters in mean values for each biochemical variable during the sampling period, the paired comparison design was applied to the Wilcoxon's signed-ranks test (Sokal and Rohlf 1981). The Kendall's rank correlation coefficients, τ, were calculated to test the strength of association among environmental parameters and oyster components.

RESULTS

Environmental Conditions

Monthly mean water temperatures at the two stations are given in Figure 2a. The water temperatures showed very similar seasonal cycle, with maxima of approximately 26.5 °C in summer and minima of approximately 6.5 °C in winter. Differences in temperature and salinity were not significant between stations. Salinity maxima of approximately 34 psu were recorded during late winter-early spring in both years at the two stations (Fig. 2b). Salinity minima were observed in summer in both years. During June through August 1996, the values at Station Osu were lower than approximately 32 psu at station Josan and a minimum salinity of 30 psu was recorded in Station Osu in July 1996.

SPM concentrations varied from 3.2 to 30.2 mg L^{-1} and extremely high concentrations more than 20 mg L^{-1} were observed in February 1996 and August 1997 at both stations (Fig. 2c). No correlations between SPM and chlorophyll *a* concentrations were found. Chlorophyll *a* concentrations showed several peaks throughout the year, but they showed maxima in spring (Fig. 2d). The mean of experimental period was 1.43 ± 0.79 (SD) μg L^{-1} at Station Osu and 2.48 ± 1.23 μg L^{-1} at Station Josan. The difference in chlorophyll *a* concentrations between stations was statistically significant (Wilcoxon's signed-ranks test, Osu mean = 4.63, Josan mean = 7.43; $0.001 < P < 0.01$).

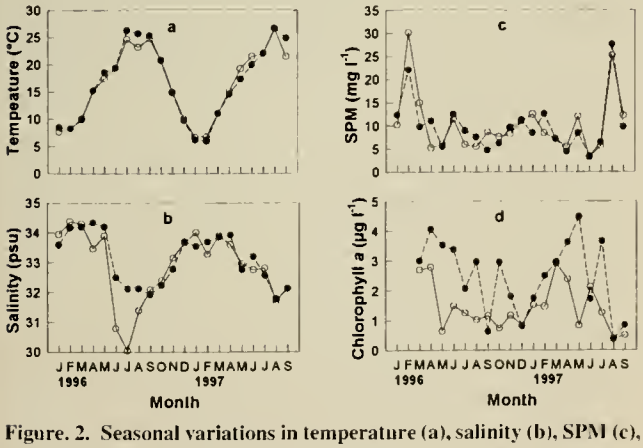

Figure. 2. Seasonal variations in temperature (a), salinity (b), SPM (c), and chlorophyll *a* at Stations Osu (open circle) and Josan (black circle) from January 1996 to September 1997.

Biometry and CI

Biometric measurements showed that there was no growth in shell length of oysters during the sampling period. Mean shell lengths ranged between 68.9 and 95.6 mm at Station Osu, and between 70.7 and 83.5 mm at Station Josan. Mean fresh and dry tissue weights showed an apparent seasonal variation with maxima in spring. Mean dry shell weight ranged between 15.620 and 30.861 g at Station Osu, and between 13.247 and 32.772 g at Station Josan. The mean dry shell weight over the sampling period at the two stations was 22.496 g.

The seasonal variations of CI were very clear at both stations (Fig. 3). The maxima in CI were in April to May in both years and were followed by an abrupt decline between June and August. At Station Josan, a subsequent rapid increase in CI values was observed in November 1996. However, at Station Osu, the minimum value of 43 in August 1996 remained constant during the autumn-winter period and a slow recovery occurred in February 1997, 3 mo after the recovery in November 1996 at Station Josan. In both years the CI maxima were much higher at Station Josan than at Station Osu. The maximum CI values at Station Josan were 104 and 133 in 1996 and 1997, respectively, whereas the values at Station Osu did not exceed 90.

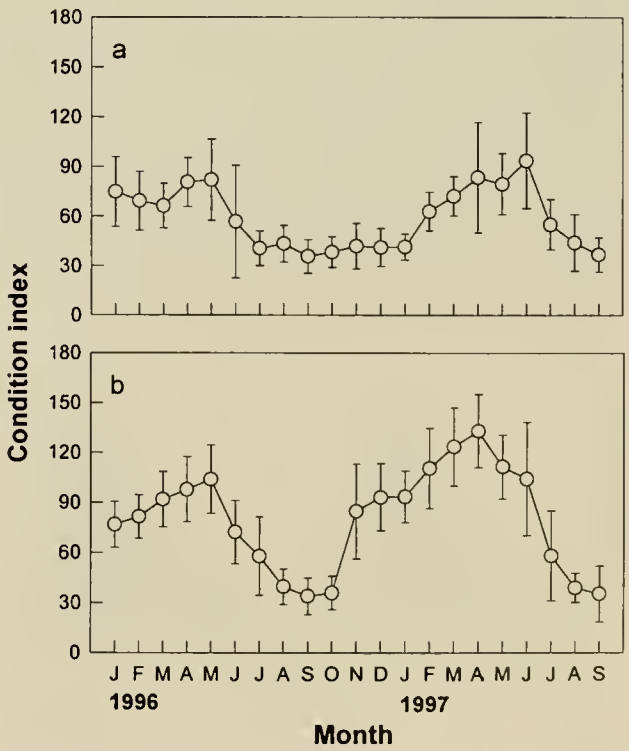

Figure. 3. Seasonal variation in CI at Stations Osu (a) and Josan (b) during the experimental period.

Reproductive Activity

The gametogenic cycles of the two cultured populations in terms of GMI are presented in Figure 4. Since differences between male and female were not considered in the analysis of the biochemical composition, GMI is here presented as the means of pooled data from both sexes. There was good agreement in the seasonal cycles of GMI between stocks. Gonadal tissue development started in November and December and the GMI increased progressively until June. The GMI maxima were recorded from June to August when CI decreased sharply. Planktonic larvae of oysters were observed throughout the summer from June to September in both 1996 and 1997. These results indicate that spawning activity continued during the summer period. The maxima were then followed by a sharp decline due to spawning, which ended in September.

Tissue Weight of a Standard Animal

Figure 5 shows seasonal variations in dry tissue weight for a standard animal (dry shell weight = 22.496 g). There were remarkable seasonal variations in dry tissue weight in each stock and the patterns were similar to those of CI, with peaks in April and May, decreases during summer, and minima in early autumn. The amplitude was greater at Station Josan. Therefore, with the exception of the time of minimum dry tissue weight in summer, standard animal from Station Josan had significantly ($P < 0.001$) higher dry tissue weight than that from Station Osu. At Station Josan, substantial increment just after the times of minima in dry tissue weight was initiated in November 1996. However, at Station Osu, no increase in dry tissue weight was found during late autumn-winter 1996.

Biochemical Composition

Seasonal variations in mean percentage of almost all the biochemical components showed clear seasonal trends and the patterns of each component were similar between stations. Protein percentages ranged from 40.5% to 66.8% of the dry tissue weight, with maxima in summer when the dry tissue weights were minimal at both stations. Lipid percentages were slightly higher in spring than the rest of the year. The values fluctuated between 2.5% and 11.6% at both stations. In spite of significant differences in dry tissue weight, no differences in the mean percentage compositions of protein, lipid, and water showed between stations. Mean carbohydrate (also glycogen) percentage was however significantly higher at Station Josan than at Station Osu (Wilcoxon's signed-ranks test, Osu mean = 7.00, Josan man = 11.67; $0.001 < P < 0.01$), with the values from 3.5% to 24.9% (average 12.0%) at Station Osu and from 2.2% to 33.0% (average 16.8%) at Station Josan. The levels were negatively correlated to the protein levels, with maxima in the winter-spring season. Glycogen levels accounted for most of total carbohydrate levels so that their seasonal variations paralleled those of carbohydrate. Ash levels showed a slight inverse relationship with carbohydrate levels, with minima values of 11.0% and 9.5% in May 1996, and maxima of 23.0% and 17.0% in September 1996 at Stations Osu and Josan, respectively. Mean ash percentage was higher at Station Osu than at Station Josan (Wilcoxon's signed-ranks test, Osu mean = 11.06, Josan mean = 5.50, $P < 0.001$). Water content ranged from 74.5% to 88.4% and from 78.3% to 87.2% of the fresh tissue at Stations Osu and Josan, respectively.

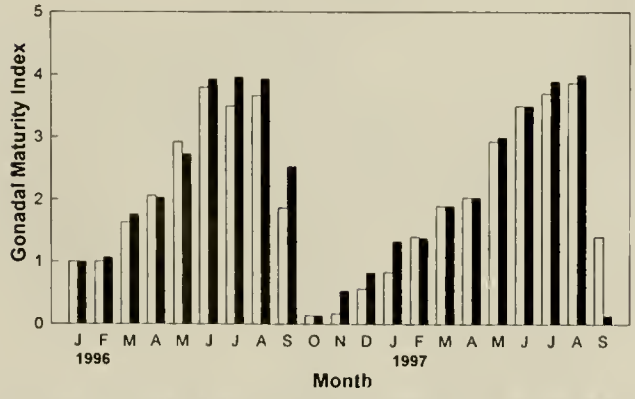

Figure. 4. Seasonal variation in gonadal development, as Mann's gonadal maturity index (1979) at Stations Osu (open bar) and Josan (black bar).

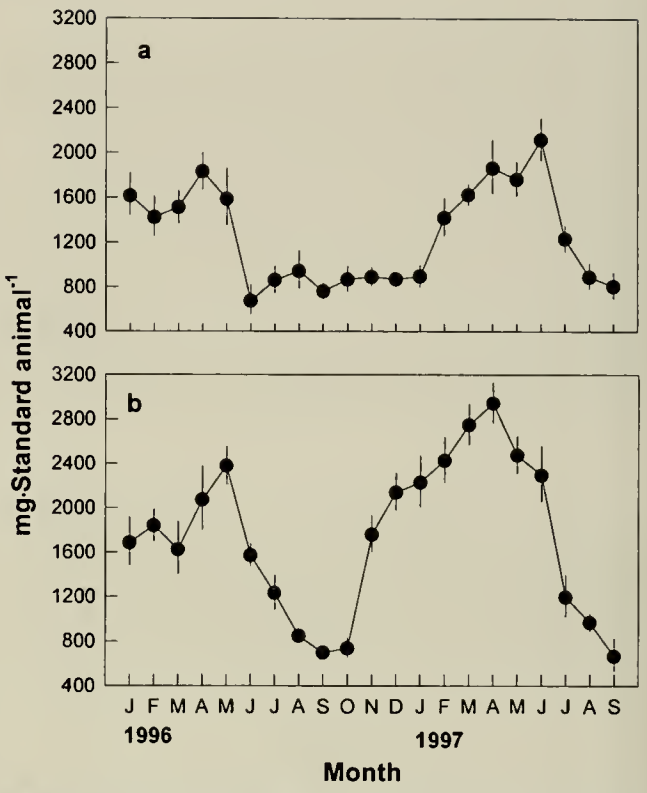

Figure. 5. Seasonal variation in dry tissue weight in standard animal of 22.496 g in dry shell weight at Stations Osu (a) and Josan (b). Vertical bars represent 95% confidence intervals.

The absolute values of biochemical components for a standard animal, calculated from the percentage composition and the dry tissue weight (Fig. 5), are presented in Figure 6 as milligrams per standard animal. In addition, correlations between the environmental parameters and the oyster components observed during the sampling period are summarized in Table 1. GMI of oysters was strongly correlated to temperature ($0.01 < P < 0.001$), but carbohydrate (also glycogen) values of the standard animal were negatively correlated to temperature at both stations. Kendall's rank correlation matrices show that the accumulation and depletion cycles of storage or reserve materials are different between stations. At station Osu both the mean CI and the standard animal dry weight were significantly correlated to its protein and lipid values

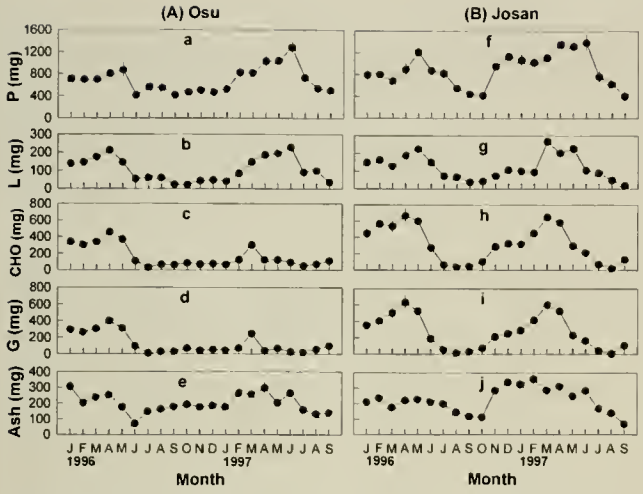

Figure. 6. Seasonal variation in weights of biochemical components in standard animal of 22.496 g in dry shell weight at Stations Osu (A) and Josan (B). P, protein; L, lipid; CHO, carhohydrate, G, glycogen. Vertical bars represent 95% confidence intervals.

($P < 0.001$), weakly correlated to the carbohydrate values, but not to the glycogen values. Protein values were significantly correlated to lipid levels ($P < 0.001$), but not to carbohydrate and glycogen values. However, at Station Josan, both the mean CI and the standard animal dry weight were significantly correlated to almost all biochemical components. Protein values were significantly correlated to lipid values ($P < 0.001$) and also, though weakly, to carbohydrate and glycogen levels ($0.05 > P > 0.01$). The differences between stations are attributed to the lack of accumulation of carbohydrate (largely glycogen) from the autumn period of 1996 to the end of the study at Station Osu (Fig. 6, c and d). Significant differences in mean values for protein and carbohydrate contents of the standard animal between stations were obvious from Wilcoxon's signed-ranks test (Osu mean = 4.25 and 4.33, Josan mean = 12.59 and 12.11, respectively; $0.01 < P < 0.001$ for protein and $P < 0.001$ for carbohydrate).

DISCUSSION

There were no differences in temperature, salinity, and SPM concentration between the two stations observed. However, annual mean chlorophyll *a* concentration was significantly higher at Station Josan in Jaran Bay than at Station Osu in Hansan-Koje Bay. The latter bay has been characterized by low chlorophyll *a* concentration and low primary production of phytoplankton compared to other oyster-culturing grounds in the southern coastal bays of Korea (Lee et al. 1991, Choi et al. 1997). It is well known that water movement can determine the amount of food supply available to suspension feeders. Water exchange rates in Hansan-Koje Bay are around 10% and 5% of the whole water volume of the bay during the spring tide and the neap tide, respectively (Yoo et al. 1980). Therefore, it is unlikely that the food available to the oysters is transported from the outer part. The suspended oyster-culturing grounds have been developed densely with a total area of 11 km^2 in the bay. This area corresponds to 23% of total water surface area of the bay. In relatively shallow areas with long residence time of water like Hansan-Koje Bay, filtration by a dense population of suspension feeders can make a significant impact on the phytoplankton biomass (Yoo et al. 1980, Cloern 1982, Officer et al. 1982, Loo and Rosenberg 1989). In addition, at a low flow rate of the bay, filtration rates of the oysters might be reduced by filtered water being recirculated (Riisgård 1977). On the other hand, Jaran Bay is characterized as a more or less eutrophic environment (Choi et al. 1997). Tidal current is relatively stronger because the bay is exposed directly to the open ocean.

CI showed a similar seasonal cycle, with minimum values in late summer and peaks in spring at both stations. However, CI values were considerably higher at the Station Josan than at Station Osu. Part of this difference in CI values was most likely due to difference in food availability (largely chlorophyll *a*). Of major interest in the seasonal variation of CI of the oysters from these stations was the difference in the time of initiation of CI recovery after the summer spawning between stations. After the summer spawning, CI of Station Josan oysters commenced a rapid recovery in November. However, those of Station Osu showed only a slow recovery in February 1997, 3 mo later than at Station Josan. Similar variation was recorded in the dry tissue weight of standard animal. These variations have important implications for cultivation and harvesting strategy because there is the greatest demand for oysters between December and January in Korea. Our results may imply that oysters from Station Josan are possible to market during this period, but those from Station Osu are not suitable for marketing at the same time.

The reproductive cycles of the oysters were similar between stations. Gametogenisis was initiated at 10 °C at Station Osu and 15 °C at Station Josan in late autumn. The temperature range at which initiation of gametogenesis and spawning in *C. gigas* occurs is well summarized by Ruiz et al. (1992, Table IV of that work). In the study areas from May to October, the water temperature exceeds 18 °C to 20 °C, which is suggested as a minimum temperature required to induce spawning (Mann 1979). The cycles of gametogenisis and CI (also dry tissue weight of standard animal) indicate that the breeding period of *C. gigas* from these areas is extended over several months during the summer-early autumn period, similarly to the case of New Zealand (Dinamani 1987). The precise timing and intensity of spawning is difficult to determine from a monthly sampling strategy. However, although a similar seasonal cycle in gametogenisis between stations was recorded in this study, the amplitude of seasonal fluctuation of CI was much greater in Josan oysters than in Osu ones. Park et al. (1999) showed that from the greater fluctuations of various CIs in Station Josan, spawning intensities are stronger in the Josan oysters than in the Osu ones. They also concluded that based on the measurements of every 10 days for 4 mo (June to September 1997), both abundance and lipid content of D-shaped larvae are much greater at Station Josan than at Station Osu, and thus the spawners are physiologically more healthy at the former station. Such a difference between stations is probably a major factor in explaining the reason why seeds of oysters are not settled and collected on ropes in Hansan-Koje Bay.

The reproductive strategy of *C. gigas* can be considered an adaptation to ambient environmental factors such as mainly temperature and nutritional conditions (Lubet 1976, Ruiz et al. 1992). For bivalve populations that experienced a similar thermal regime, the availability of food in the environment and thereby the levels of nutrient reserves within the animals directly support gonadal growth and reproductive cycle (Giese 1969, Gabbott 1975, 1983, Bayne 1976, Bayne and Worrall 1980, Newell et al. 1982, Rodhouse et al. 1984, Bricelj et al. 1987). Therefore, local variations in SI, dry tissue weights, and spawning activities recorded in this

TABLE 1.

Kendall's rank correlation coefficient matrix for temperature (T), salinity (S), chlorophyll *a* (CHL), Gonad Maturity Index (GMI), Condition Index (CI), and dry tissue weight (DTW), protein (P), lipid (L), carbohydrate (CHO), glycogen (GLY), and ash content of a standard animal.

	T	S	CHL	GMI	CI	DTW	P	L	CHO	GLY	Ash
					Station Osu						
T		−0.67***	−0.33*	−0.50**	−0.19	−0.17	−0.11	−0.06	−0.41**	−0.34*	−0.43**
S	−0.53***		0.32	−0.38*	0.24	0.35*	0.18	0.21	0.43**	0.36*	0.44**
CHL	−0.16	0.59		0.30	0.35*	0.31	0.25	0.31	0.18	0.02	0.40*
GMI	0.43**	−0.21	−0.01		0.30	0.13	0.21	0.29	−0.07	−0.15	−0.25
CI	−0.43**	0.54***	0.05	−0.01		0.81***	0.69***	0.75***	0.51**	0.26	0.39*
DTW	−0.32*	0.49**	0.06	−0.02	0.91***		0.76***	0.72***	0.38*	0.20	0.57***
P	−0.31	0.36*	0.10	0.09	0.73***	0.79***		0.66***	0.26	0.06	0.46**
L	−0.33*	0.53***	−0.14	0.06	0.64***	0.65***	0.56***		0.41**	0.19	0.37*
CHO	−0.60***	0.83***	−0.19	−0.19	0.55***	0.51**	0.32*	0.64***		0.76***	0.42**
GLY	−0.59***	0.80***	−0.18	−0.15	0.56***	0.53***	0.34*	0.60***	0.96***		0.37*
Ash	−0.57***	0.39*	−0.04	−0.16	0.70***	0.72***	0.71***	0.47**	0.40*	0.40*	
					Station Josan						

* $0.05 < P < 0.01$; ** $0.01 < P < 0.001$; *** $P < 0.001$.

study can be expected to result from differences in accumulation and utilization of storage or reserve materials.

The seasonal cycles in dry tissue weight of standard animal reflected the reproductive cycle with the weights showing maxima prior to the summer spawning and abrupt declines during spawning. The difference in the strength of seasonal weight fluctuations between stations (Fig. 5), along with that in CI fluctuations, indicates that the intensity of the spawning is different between stocks. Although there was a general tendency that the seasonal variations in the absolute values of biochemical components paralleled those of dry tissue weights (Fig. 6), the seasonal patterns of carbohydrate and glycogen contents differed distinctly between stations. In Josan oysters, a rapid recovery of glycogen values began in November 1996 after the abrupt declines during spawning in summer (June to September 1996) and peaked in April 1997. However, the accumulation of carbohydrate and glycogen reserves in Osu oysters (although there was a small peak in March 1997; Fig. 6) was not observed from the autumn period of 1996 to the end of the study.

The accumulation of glycogen reserves appears to be related to the time of maximum phytoplankton biomass (Ansell and Trevaillion 1967, Ansell 1972). Glycogen reserves have been considered to be the main energy reserves both for the formation of gametes of marine bivalves, especially under conditions of nutrient stress and also for the maintenance during nutritional stress (Beninger and Lucas 1984 and refs. therein). Thus the lack in the accumulation of glycogen at Station Osu presumably resulted from insufficient food availability within the bay and this might allow Osu oysters to lead to low gamete proliferation. Over the study period the absolute carbohydrate and glycogen values were considerably higher in Josan oysters than in Osu ones. Deslous-Paoli and Héral (1988) reported a similar pattern in *C. gigas* from Marennes-Oléron Bay (France). These authors reported that the levels of glycogen were less than 5% of dry tissue weight in the oysters overstocked with high cultivated density, whereas the levels were more than 10% of dry tissue weight in oyster ponds with low density of oysters in the same area. They also showed that a deficiency of food due to an overstocking can disturb the physiology, particularly the processes of gametogenesis and spawning, inducing the failure of spat settlement.

Glycogen is transformed into lipid for the formation of gametes (Gabbott 1976, Lubet 1976). Lipid reserves are lost in spawning of adult female bivalves (Gabbott 1983). The variation in the absolute values of lipid in the oysters from this study supports these hypotheses, with maxima prior to spawning. Since protein constitutes the major organic component of bivalve oocytes (Holland 1978), protein maxima prior to spawning are reasonable. Protein also serves as an energy reserve during gametogenesis (Mann and Glomb 1978, Adachi 1979, Barber and Blake 1981) and during energy imbalance (Gabbott and Bayne 1973, Beninger and Lucas 1984). However, since gonadal development is an energy-demanding process and the oysters in this study had only a very short gonadal resting stage, it was difficult to assess the role of each biochemical component as maintenance energy during the energy imbalance period. Furthermore, the filtration and ingestion rates decrease above 20 °C (Le Gall and Raillard 1988), whereas oxygen consumption rates increase (Bougrier et al. 1995). There may be therefore a synchrony in the energy-required timing for maintenance and spawning during summer. Riley (1976) found that from a controlled starvation experiment of *C. gigas*, lipid and protein were the main energy reserves. Whyte et al. (1990) concluded that protein contributed more than carbohydrate to maintenance energy in oysters under conditions of extended food deprivation, even when carbohydrate was apparently available in sufficient quantity. The seasonal variation patterns in the absolute values of lipid and protein were similar between stations, but the protein value was significantly greater in Josan oysters than in Ous oysters. A difference in the accumulation timing of these reserves after spawning was found similar to that in the dry tissue weight (Figs. 6 and 4).

Apparently, histological examination showed that gametogenesis of the oysters observed was initiated simultaneously with the accumulation (Station Josan) and with a minimum level (Station Osu) of reserve materials in late autumn-early winter. Then gamete development continued during the period of increase in reserve materials throughout the spring and maximum levels in almost all the biochemical components occurred at the moment of ripeness in late spring, followed by a subsequent rapid decrease during spawning (June through September). This type of bivalve may be considered to be an opportunistic species (see definition in "Introduc-

tion") that have a direct dependence on food availability in its ambient environment. This finding is inconsistent with the results of Ruiz et al. (1992) that for *C. gigas* in suspended culture in El Grove (Galicia, Spain), the glycogen stored is used in the gametogenesis and the protein and the lipid are utilized in winter when available food is scarce. This discrepancy will have to be explained by various endogenous and exogenous parameters.

In conclusion, the intensity of gamete proliferation and the condition of the oysters in suspended culture in Korean waters seem to be largely determined by the nutritional conditions in ambient environments. Food availability is considerably restricted in the semi-enclosed bay system with the high density of oysters. In such a restricted nutritional condition, the accumulation of reserve materials, particularly glycogen and protein, is expected to be insufficient to meet the energy required for increasing gonadal development and tissue weight. Higher seasonal fluctuation in CI and dry tissue weight of the oysters cultivated in the outer open system suggests that they experience a superior nutritional condition, with much greater contents in reserve materials. As a result, our findings indicate that the outer open system is a more suitable site for the suspended culture to procure enough wild seeds and condition of oysters. These results also conclude that for the enclosed bay system like Hansan-Koje Bay, the cultivated density of oysters must be readjusted, taking carrying capacity of the bay into consideration (Héral 1993).

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THE TRANSMISSION OF MICROSATELLITE ALLELES IN AUSTRALIAN AND NORTH AMERICAN STOCKS OF THE PACIFIC OYSTER (*CRASSOSTREA GIGAS*): SELECTION AND NULL ALLELES

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ABSTRACT Variation, transmission, and selection at 24 microsatellite loci are studied in five experimental families of the Pacific oyster (*Crassostrea gigas*). Two families are from naturalized North American stocks, and three come from Australian stock. As expected, there are multiple alleles at these loci and their segregating variation is reduced to four alleles or less in full sib progeny groups. Two to 21 loci were tested per family. Eight of the 24 loci have only codominant alleles, but 16 loci also have non-amplifying or null alleles. Of the 172 (43 × 4) parental sequences that were progeny tested, 30 (17%) were null alleles. Null alleles segregate in both Australian and North American stocks and their presence is heterogeneous among crosses. Overall null allele frequency in North American crosses was estimated to be 11% (eight of the 72 alleles progeny tested), just significantly less than the 22% (22 of the 100 alleles progeny tested) in the Australian stocks ($P = 0.04$). After accounting for nulls in genetic hypotheses, selection in the form of significant deviations from Mendelian expectations is observed in 16 of 43 progeny tests (37%). There is no systematic association between null alleles and selection, but analysis of dominance by sequential *G*-tests reveals non-additive kinds of zygotic selection. This has also been recorded in two other oyster species and the blue mussel. It appears that null alleles at microsatellites and selection near genetic markers are expected phenomena when studying transmission of genetic markers in bivalve molluscs. The implications of these results for breeding, aquaculture, and population genetics are discussed.

KEY WORDS: Pacific oyster, SSLP, genetic marker

INTRODUCTION

The aquaculture production of bivalves (including oysters, mussels, scallops, and clams) in 1997 was valued at some 8 billion dollars world-wide, and constituted some 7 million metric tons of food (FAO 1999). Production of Pacific oysters (*Crassostrea gigas*) in the same year was nearly 3 million tons; it is the most widespread, cultivated invertebrate on earth (Shatkin et al. 1997, FAO 1999). A small, but growing fraction of the global production of Pacific oysters is based on hatchery stocks. For example, all Australian production comes from hatchery crosses followed by nursery rearing and then grow-out. Selective breeding programs for the Pacific oyster in Australia (Ward et al. 2000) and the U.S.A. (Hedgecock et al. 1997) aim to produce broodstock with improved domestication qualities. To assist in these programs, we have begun to apply a suite of microsatellite loci. These, in association with other genetic markers including allozymes and AFLPs are used in linkage mapping, trait mapping, pedigree analysis, and marker-assisted selection.

Previous work on inbred families of the Pacific oyster revealed substantial segregation ratio distortion at allozyme loci (Foultz 1986a, McGoldrick 1997). In addition, a heterozygosity growth rate correlation and heterozygosity deficiency is observed at the population level for bivalves in general (see Gaffney 1994) and was first observed in the American oyster *Crassostrea virginica* (Singh and Zouros 1978). There is a suggestion from the study of inbred lines in Pacific oysters that particular allozyme alleles might be linked to genetic regulatory backgrounds that segregate in families and influence the ratio distortion for particular alleles at many loci simultaneously (McGoldrick and Hedgecock 1997). Here we examine whether distortion is occurring at microsatellite loci in inbred and outbred crosses, and the nature of the distortion (e.g. dominant, overdominant, or underdominant) when it occurs.

In oysters, detailed studies of microsatellite transmission are few. Naciri et al. (1995) describe inheritance patterns for three loci in the European flat oyster, *Ostrea edulis*. Fewer than 20 progeny were examined for each of two single-pair crosses; parental genotypes were inferred. Two loci gave progeny ratios that accorded with Mendelian expectations. The third locus gave two homozygote classes, but no heterozygotes in one family, and a large heterozygote excess in the other family. A cross between a heterozygote for the two amplified alleles and a null homozygote could explain the first result. For the second, the authors suggest that lethal alleles hitchhiking with amplified alleles could be responsible. A second flat-oyster paper (Bierne et al. 1998) examined four microsatellite loci in about 80 larvae and post-larvae from two full-sib crosses. Null alleles were not reported, but deviations from Mendelian expectations were recorded in about one-half of the cases.

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Null alleles are not uncommon in single locus, PCR-based typing in neutral DNA. Nulls frequently reflect changes in one of the two PCR priming sites that prevent a primer from binding efficiently, thus blocking amplification during the polymerase chain reaction. Examples where mutations are known to produce null alleles include a single-basepair transversion (Egglestontott et al. 1997), a 1-bp insertion (Band and Ron 1997), a 4-bp deletion (Jones et al. 1998), and an 8-bp deletion (Callen et al. 1993). More commonly, the existence of null alleles is inferred at the population level when observed heterozygote frequencies are less than those expected at Hardy-Weinberg equilibrium (e.g. Foltz 1986b). In such circumstances the frequency of the null allele may be estimated as that which minimizes deviations from equilibrium. However, we use breeding data to provide more formal proof of the existence of a null allele. Null alleles, once recognized, are manageable in family studies, but somewhat less so in population studies (see Callen et al. 1993). Once identified, null alleles can be treated as recessive alleles, while amplified microsatellite alleles are scored as codominant alleles. The frequency of null alleles and the question of whether they are homogeneous across stocks and loci is an important issue for population genetic analysis, in determining exclusion probabilities in pedigree analysis, and in marker-assisted selection.

In this study we report the results of segregation tests of 24 microsatellite loci in five crosses of Pacific oysters. Null alleles are present at many loci, but even when these are allowed for, deviations from Mendelian proportions are frequently observed. The forms of selection in other published studies of bivalves are also investigated and the implications of these observations are discussed.

MATERIALS AND METHODS

Microsatellite Loci

Seventeen of the 24 informative microsatellite loci (*ucdCg1. . .ucdCg28*) were developed at University of California, Davis (McGoldrick 1997). Seven other informative microsatellite loci (*cmrCg2. . .cmrCg151*) were developed at CSIRO Marine Research, Hobart. Primer sequences and available GenBank accession numbers are reported (Table 1).

Genomic Amplification

Genomic DNA template for North American samples was prepared for amplification utilizing a small biopsy of adult mantle tissue and a standard phenol chloroform method (Ausubel et al. 1994) and was typed at the University of California at Davis Bodega Marine laboratory. Loci were amplified in 96-well sample plates. After optimization of a sample of five loci (after Cobb and Clarkson 1994), a 5-μL consensus reaction cocktail was established (2 mM $MgCl_2$, 300 μM dNTP mix with a 1:100 dilution [v/v] of Dupont Renaissance™ tetramethylrhodamine-6dUTP in 10 μM stock dNTP, 5 pM of primer, 50 ng template, and 0.04 units *Taq* polymerase from Promega Corporation [Madison, WI]). A primer set was not accepted unless it could generate scoreable phenotypes with the consensus cocktail. Samples were amplified in a Bio-Oven III thermocycler (Biotherm Corporation, Fairfax, VA) with an initial denaturation at 92 ° for 2 min followed by 35 cycles of 92 ° for 30 sec, 55 ° for 30 sec, and 72 ° for 30 sec. Following amplification, 4 μl of formamide loading dye (10 mL formamide, 100 μL 0.5M EDTA, and 2 mg bromphenol blue) was added to each well and the samples were denatured at 92 ° prior to

TABLE 1

Primer sequences flanking Pacific oyster microsatellites.

Locus	Clone	GENBANK	5′-Forward (labeled)	5′-Reverse
ucdCg01	AE27	—	CAAGCTTAAAAAAGCAAGTTTACG	TGCGGTGCTATTATGAACCA
ucdCg02	AM86	—	TTGCAGGAAGCAAGAGATGA	CTTGTTAACTGCCGGTGAGG
ucdCg03	AS88	—	GTTTGAACCCATGCAGAGGT	CAGAACTTTTGAGAGAGAGAGAGA
ucdCg04	BR09	AF051170	ATAATAATTAAAGGGGTTAAGGGG	GTGGTAGCAATTGTGTCCTATG
ucdCg05	BS55	–	GGGGTCTGTGATATCGGAGA	GGTTCTACGCACAGTGCTGA
ucdCg06	BV59	AF051172	AAGCAACTATCAGTTTTTGGTAGC	AATGAGCTGACAGTTCATAGGC
ucdCg08	CE48	AF051175	CTTCTCACTTCACACACTCATCC	TTTAAACTTGTGTAAAGCATCTGG
ucdCg09	CF08	–	TTAAACTTGTGTAAAGCATTTGG	CGTTCATCGATTTTCGCAAT
ucdCg10	C175	–	TGCACCAATTTGAGATGTGA	ACTGAGTTTGAAAATGTCACCG
ucdCg12	CK90	–	TTCAAAACGACATAGTCCACA	ATCTGAGCTTGCATGGGAAC
ucdCg13	CU03	AF051179	TGTGTAAATCAACAAAAGAAATGC	GATCAATAATTTTCATGCCAGA
ucdCg14	BY56	AF051174	GGTGAAGGAAAACACAAAACA	TTAGCTGCCGCTCAAGTTTT
ucdCg15	S72	–	TGATGCAGTAAGATTTCATTTCA	ACAGGTAACCCCCTCCACTC
ucdCg18	AP46	–	TCCATGTTTACTGCTACTTTTGG	AAATGCTGTGCAGAGAAGCC
ucdCg21	BQ12	–	GCCCTCTAAATTAAAATCTCTCT	CCGCCATAGGTTTGAAAATT
ucdCg22	BS11	–	CCCCAACTCAAACAGACGTT	TAGTCAGACGTTCCTAACTCTTCG
ucdCg28	CQ72	AF051178	TGTTTAATGATGTGTACCGCG	ATCAAATTGGCTGTATTTACAGTG
cmrCg02	C131	AF201461	AGGAGATCATATCATAAGGAGACAGAG	ATGTTTTACATTCTTACAGGTCATTCA
cmrCg03	F034	AF201462	CTTTGCCTGTGATAACACTACGTATCG	TTAAATTTCATTGACAATTATGGTCCCA
cmrCg06	H105	AF201465	ATTGTTTGCCGATACTGAGAGA	CTGACTGAAACTGCTTTGTTGA
cmrCg61	–	AF204062	GATTGGTTGAAAAAATCACACG	TAACAGCAGCGCTACCATGC
cmrCg141	–	AF204060	ACCATTTGCACCTTTCCAAC	TGACACTTGAAGCCTTGCAC
cmrCg143	–	AF204061	CTTGCCATATTGCCATGTGT	CTTTTACATGGAATTGTCACAGG
cmrCg151	–	–	TGCTTCATTGTTTGTTGTATGG	CATACACAAATTGCACTTATAGCA

electrophoresis. A total of 4 μL of dye-reaction mix was electrophoresed for 1.5 h at 60 watts on an 8% acrylamide (19:1, acrylamide:bis-acrylamide) gel containing 8 M urea and 1× TBE and visualized using an FMBIO digital scanner (Hitachi, San Francisco, CA). Alleles were not sized.

Australian samples were examined at CSIRO Marine Research in Hobart, Tasmania, Australia. Extractions of whole body tissues were performed from 198-day-old juveniles and muscle tissue from 395-day-old adults. Loci were amplified in 96-well trays in a Perkin Elmer 9600 thermocycler using 5′- HEX, FAM-, or TET-labeled primers (PE Applied Biosystems, Foster City, CA.). A consensus reaction chemistry was again established in the absence of fluorescent dNTP. Reaction cocktails consisted of 2 mM $MgCl_2$, 200 μM dNTP, 1 pM of primer, ~10 ng template, and 0.02 units *Taq* polymerase (Promega Corporation). Samples were sized and genotyped using an ABI377 sequencer with Genotyper software (PE Biosystems).

Microsatellite loci with smeary, faint, or complex electrophoretic banding patterns are not reported here. Two contaminating individuals, one in family 97-2, and one in family 93IL2 were removed from analysis according to criteria described previously (McGoldrick and Hedgecock 1997).

North American Oysters

Two inbred lines derived from selfed hermaphrodites (89-6 and 89-7) were crossed on June 29, 1993 to generate four genotypic populations: 6 × 6 , 6 × 7, 7 × 6, and 7 × 7 (Hedgecock et al. 1995). Approximately 1 year later (340 days post-fertilization), a hybrid F_2 population was made by sib-mating a male and female from a 7 × 6 F_1 hybrid line. Two hundred progeny were labeled in November (203 days post-fertilization) with a numbered bee tag attached with cyano-acrylate adhesive and set out on a lease owned by Hog Island Oyster Co. (Tomales Bay, CA). After about 1 year, labeled animals were brought back to Bodega Marine laboratory and held for DNA analysis. Twenty-one animals died prior to DNA extraction.

In another experiment, two unrelated oysters were crossed to generate an F1, and two F1 sibs were mated in 1993 to generate line 93IL2. The 72 progeny examined were thus 25% inbred. Hatchery and grow-out protocols are described in Breese and Malouf (1974).

Australian Oysters

Six Australian lines derived from pair-crosses of unrelated commercial broodstock were created on January 8, 1997. Animals were grown in 140-L containers, settled, and stocked to an upwelling nursery system according to commercial practice. On May 8, 1997, animals were transferred to Duck Bay (Smithton, Tasmania) where they were grown at low density in sectionalized seed trays. Family 97-2 was examined for 21 microsatellite loci. Forty-one oysters were sampled at day 198 post-fertilization and typed for 18 microsatellite loci (see Table 5). Ten of these loci plus three other loci were examined in a supplemental sample of 31 older adult animals taken on day 359. Four loci were typed in families 97-1 and 97-6 (see Table 6), from which samples of 39 and 41 juveniles were taken on days 198 and 359, respectively.

Tests of Mendelian Segregation

Mendelian segregation is tested with *G*-tests (Sokal and Rohlf 1995). Initially, segregation ratios are assumed to be derived from neutral, Mendelian, codominant, and observable alleles. Secondarily, null alleles are allowed to modify hypothesis testing. When multiple hypotheses exist within a family, then all consistent hypotheses are considered within the family. For the purposes of segregation tests, *G*-statistics are calculated, ranked in order of decreasing probability, and the test with most likely ratio is taken to be the correct one for the purposes of tabulation.

To uncover whether distorted segregation ratios indicate gametic incompatibility, meiotic drive, or viability selection, we use partition *G*-tests for gametic and zygotic selection (Pham et al. 1990, Lorieux 1995). First, tests of the prior Mendelian hypothesis are made. If this test is significant, estimates of allele frequencies are made outside of the prior hypothesis and the genotypic proportions are tested given these allele frequencies. Residual significance indicates zygotic selection. Thirteen cross types are recognized with various levels of informativeness. The informativeness of *G*-tests for 13 unique types of segregation at a single locus allowing for null alleles is shown in Table 2. Type 7 (or di-hybrid) crosses are tested for 1:2 ratio of the most frequent homozygote class relative to the heterozygote (a test of deleterious recessive gene action linked to the less frequent homozygote). In addition, a 1:1 ratio of the two homozygote classes is also tested to indicate heterozygote advantage or disadvantage, again using *G*-tests. Collectively, these tests are used to propose the simplest form of selection that could produce the observed ratio.

For the purposes of tabulation we adopt a notation for presenting selection tests in tables. Allelic selection against parental alleles is underscored in the appropriate parent and resulting genotypes e.g "*A*/*B* × *C*/*D*" in the parents indicates selection against the transmitted "*A*" allele and "*A*/*C*, *A*/*D*, *B*/*C*, *B*/*D*" in the progeny indicates that selection against the "*A*" allele results in deficiency in progeny classes containing the "*A*" allele. Zygotic selection on genotypes is indicated by set bracketing of specific genotypes in progeny tests, e.g., "{*A*/*B*}". When zygotic and allelic selection overlap in the same genotype, zygotic selection is indicated by set brackets and alleles are underscored for the purposes of tabulation, e.g. "*A*/*B* × *C*/*D*" in the parental fields and "*A*/*C*, {*A*/*D*}, *B*/*C*, *B*/*D*" in the progeny indicates selection against the "*A*" allele plus further residual zygotic selection against the "{*A*/*D*}" genotype. When useful in tabulation, genotypic classes with undistinguishable parental alleles are indicated with parentheses with any alleles requiring distinction due to selection contained within as before, e.g. "*A*/*O*, × *B*/*A*" in the parental fields and "*A*/*B*, (*A*,*O*), *B*/*O*" in the progeny indicates selection associated with the "*O*" null allele but not the hidden "*A*" allele. When two null alleles are present that are distinguished by their transmission, the second null is indicated " *O*′ " (see Tables 3–6 and Appendix 1).

RESULTS

There are 43 informative tests of Mendelian segregation plus four tests to confirm fixed transmission of homozygous alleles. The number of loci tested, presence of nulls, and forms of selection (after accounting for nulls) is presented in two North American and three Australian families.

Family 1 (7 × 6 – Hybrid Progeny from North America)

Eight loci were tested (Table 3). The significance level for rejecting Mendelian segregation is therefore set at $1 - (1 - 0.05)^{1/8} = 0.0064$. A null allele is postulated in the female parent for

TABLE 2

Types of crosses when null alleles are segregating in a population.

Genotypes				Components for tests of distortion			
Parent 1	× Parent 2	Type	H_0	$G_{Parent1}$	$G_{Parent2}$	$G_{Gamette}$	$G_{Zygotic}$
AO	*OO*	0	1:1	√		√	
AO	*AO*	1	3:1				√
AB	*OO*	2	1:1	√		√	
AB	*AA*	3	1:1	√		√	
AB	*CC*	4	1:1	√		√	
AA	*BO*	5	1:1		√	√	
AA	*BC*	6	1:1		√	√	
AB	*AB*	7	1:2:1			√	√
AB	*AO*	8	2:1:1	(√)[a]	(√)	√	(√)
AO	*BO*	9	1:1:1:1	√	√	√	√
AB	*CO*	10	1:1:1:1	√	√	√	√
AB	*AC*	11	1:1:1:1	√	√	√	√
AB	*CD*	12	1:1:1:1	√	√	√	√

[a] Partial information indicated by parentheses.

ucdCg01, giving the dam the phenotype M and the genotype *O/M*. This permitted the appearance of F phenotypes (*O/F* genotypes) in the progeny; the sire having the *S/F* genotype. Progeny numbers then accorded with Mendelian expectations given the adjusted significance level.

Four of the eight loci do not deviate significantly from Mendelian expectations. Of the four that do deviate, three (*ucdCg04*, *ucdCg08*, and *ucdCg10*) show a heterozygote deficiency due to zygotic and apparent underdominant selection. One locus (*ucdCg13*) shows a deficit of one homozygous class and an excess of the other homozygous class, apparently due to a deleterious effect linked to the *F* allele transmitted by one of the parents (see Table 7).

Family 2 (93IL2 – 25% Inbred Progeny from North America)

Ten loci were tested (Table 4). The significance level for rejecting Mendelian segregation is therefore set at $1 - (1 - 0.05)^{1/10} = 0.0051$. Null alleles are postulated for five loci. One locus (*ucdCg09*) has one parent with a F phenotype and one with a S phenotype, but gives S, F, S/F, and null phenotypes in offspring; in this instance the parents are presumed to be *O/S* and *O/F* genotypes. In two other loci (*ucdCg10* and *ucdCg14*), a null allele is postulated for either the male or female parent to permit the observed progeny phenotypes. Locus *ucdCg15* is ostensibly a cross between an F phenotype sire and a null homozygote dam, but it yielded F, S, F/S, and null phenotypes. It is presumed that the sire has an *O/F* genotype and the dam has an *O/S* genotype, but that the *S* allele failed to detectably amplify in the female parent. The fifth locus was *ucdCg28*. This cross has an S sire and an F/VF dam, and the segregation yielded four phenotype classes in the progeny: S/VF, F, S/F, and VF. We postulate that the sire genotype is *S/O* and the dam genotype *F/VF*.

Six of the ten loci do not deviate significantly from Mendelian expectations. Of the four that do, *ucdCg09* shows a deficiency of the null homozygote genotype, *ucdCg14* and *ucdCg21* show deviations for all three genotypes, and *ucdCg28* shows a deficiency of genotypes containing the *VF* allele and is explained if a deleterious effect is associated with the *VF* allele.

Family 3 (97-2 – A Non-Inbred Pair-Cross from Australia)

Twenty-one loci were tested (Table 5). The significance level for rejecting Mendelian segregation is therefore set at $1 - (1 - 0.05)^{1/21} = 0.0024$. Null alleles are postulated for 12 loci. Seven loci have both parents designated heterozygous for a null allele and an amplified allele, for one locus one parent is designated as a null homozygote, and for four loci either the dam or sire is designated a null/amplified allele heterozygote.

TABLE 3

Segregation tests of microsatellite transmission in crossbred North American cross 7 × 6A.

Locus	Sire × Dam	Type	Genotypic classes in progeny				Ratio	H_0	*G*	*P*
7 × 6A:										
ucdCg01	*S/F* *O/M*	*10*	*O/F*	*:M/F*	*:M/S*	*:O/S*	27:28:46:42	1:1:1:1	7.88	0.0485
ucdCg03	*F/S* *F/S*	*7*	*F/F*	*:F/S*	*:S/S*		27:84:36	1:2:1	4.30	0.1165
ucdCg04	*F/S* *F/S*	*7*	*F/F*	*[F/S]*	*:S/S*		43:37:35	1:2:1	15.77	0.0004
ucdCg05	*F/S* *F/S*	*7*	*F/F*	*:F/S*	*:S/S*		24:68:38	1:2:1	3.47	0.1768
ucdCg06	*F/S* *S/S*	*7*	*F/S*	*:S/S*			69:77	1:1	0.44	0.5078
ucdCg08	*F/S* *F/S*	*7*	*F/F*	*:[F/S]*	*:S/S*		35:44:48	1:2:1	14.22	0.0008
ucdCg10	*F/S* *F/S*	*7*	*F/F*	*:[F/S]*	*:S/S*		58:55:39	1:2:1	15.50	0.0004
ucdCg13	*F/S* *F/S*	*7*	*F/F*	*:F/S*	*:S/S*		19:60:49	1:2:1	14.20	0.0008

$P_{cr} = 0.0064$.

TABLE 4.

Segregation tests of microsatellite transmission in full-sib mating 93IL-2.

Locus	Sire × Dam	Type	Genotypic classes in progeny				Ratio	H_0	*G*	*P*
93IL-2, Most likely hypotheses										
ucdCg01	*F/S* × *F/S*	7	*F/F*	*:F/S*	*:S/S*		12:22:17	1:2:1	1.83	0.04005
ucdCg02	*S/S* × *F/S*	3	*F/S*	*:S/S*			23:28	1:1	0.49	0.4835
ucdCg04	*S/S* × *F/S*	3	*F/S*	*:S/S*			30:22	1:1	1.24	0.2663
ucdCg09	*F/O* × [a]*O′/S*	9	*F/O′*	*:F/S*	*:S/O*	*:O/O′*	19:21:25:6	1:1:1:1	13.76	0.0033
ucdCg10	*F/S* × *O/S*	8	*F/O*	*:F/S*	*:S/–*		16:22:19	1:1:2	7.41	0.0246
ucdCg13	*F/S* × *F/S*	7	*F/F*	*:F/S*	*:S/S*		18:29:11	1:2:1	1.71	0.4260
ucdCg14	[b]*S/O* × *F/S′*	8	*F/O*	*:F/S*	*:S′/(S, O)*		25:7:20	1:1:2	13.54	0.0012
ucdCg15	*F/O* × *O′/[S]*	9	*F/O′*	*:F/S*	*:S/O*	*:O/O′*	17:18:20:15	1:1:1:1	0.75	0.8625
ucdCg21	*F/S* × *F/S*	7	*F/F*	*:F/S*	*:S/S*		9:23:32	1:2:1	18.81	0.0001
ucdCg28	*S/O* × *F/VF*	9	*S/VF*	*:–O/VF*	*:F/O*	*:S/F*	13:5:26:16	1:1:1:1	15.96	0.0012

[a] A second segregating null allele is indicated by *O′* in the dam. [b]A second segregating slow allele is indicated by *S′* in the dam. [c]The slow allele failed to amplify in the female parent, see text. P_{cr} = 0.0051.

TABLE 5.

Segregation tests of microsatellite transmission in Australian pair cross 97-2.

Locus	Sire × Dam	Type	Genotypic classes in progeny				Ratio	H_0	*G*	*P*
ucdCg02	172/201 × 172/172	3	172/172	:172/201			15:18	1:1	0.27	0.6013
ucdCg03	113/156 × 143/156	12	113/143	:113/156	:143/156	:156/156	3:13:10:4	1:1:1:1	9.53	0.0230
ucdCg04	159/159 × 159/159	–	159/159				32	1	–	–
ucdCg06	156[a]/182 × O′/166	9	156/O′	:O′/182	:156/166	:166/182	2:24:6:29	1:1:1:1	39.72	<0.0001
ucdCg08	249/332[c] × O′/272	9	249/O′	:249/272	:272/332	:332/O′	0:0:15:17	1:1:1:1	44.49	<0.0001
ucdCg09	O/214 × O′/164	9	O/O′	:O/164	:O′/214	:164/214	4:11:4:13	1:1:1:1	8.54	0.0361
ucdCg10	197/222[b] × 153/175	12	153/197	:153/222	:175/197	:{175/222}	16:14:28:5	1:1:1:1	17.95	0.0004
ucdCg12	O/93 × O/97	9	O/O	:O/93	:O/97	:93/97	6:13:2:7	1:1:1:1	9.23	0.0263
ucdCg13	148/184 × O/148	8	(O, 148)/148	:O/184	:148/184		41:3:7	2:1:1	21.87	<0.0001
ucdCg14	150/180 × 126/133	12	126/150	:126/180	:133/150	:133/180	20:6:13:8	1:1:1:1	9.69	0.0214
ucdCg15	160/171 × O/160′	8	(O, 160′)/160	:160′/171	:O/171		3:10:8	2:1:1	12.11	0.0023
ucdCg18	O/106 × 104/106′	8	(O, 106′)/106	:O/104	:104/106		17:4:10	2:1:1	2.95	0.2291
ucdCg21	134/144 × O/O	2	O/134	:O/144			9:15	1:1	1.52	0.2182
ucdCg22	229/254 × 229/254	7	229/229	:229/254	:254/254		4:21:7	1:2:1	4.01	0.1349
cmrCg02	265/265 × 265/283	3	265/265	:265/283			23:16	1:1	1.26	0.2610
cmrCg03	437/470 × 437/470	7	437/437	:437/470	:470/470		8:18:10	1:2:1	0.22	0.8946
cmrCg06	O/136 × O/136	1	O/O	:–/136			22:41	1:3	3.07	0.0797
cmrCg61	216/216 × 216/224	3	216/216	:216/224			25:13	1:1	3.86	0.0496
cmrCg141	O/186 × 186/204	8	186/204	:186/–	:O/204		13:20:7	1:2:1	1.83	0.4009
cmrCg143	O/150 × O′/145	9	O/O′	:O/145	:O′/150	:145/150	2:11:9:18	1:1:1:1	14.92	0.0019
cmrCg151	270/274 × 270/278	11	270/270	:270/274	:270/27	:274/278	13:11:5:11	1:1:1:1	4.08	0.2526

[a] The *156* allele is weakly amplifying and can be below detection, see text. [b]The 222 allele is also weakly amplified, see text. [c]The *332* allele is again weakly amplified, see text. P_{cr} is 0.0026.

TABLE 6.

Segregation tests of four microsatellites in Australian pair crosses 97-1 and 97-6.

Locus	Sire × Dam	Type	Genotypic classes in progeny	Ratio	H_0	*G*	*P*
97-1							
cmrCg61	*216/216* × *216/216*	–	*216/216*	39	1	–	–
cmrCg141	*174/204* × *O/204′*	8	*O/174 :(204′, O)/204 : 174/204′*	24:10:5	1:2:1	23.20	<0.0000
cmrCg143	*O/150* × *150/155*	8	*O/155 : (O, 155)/150 : 150/155*	14:12:13	1:2:1	5.96	0.0509
cmrCg151	*260/274* × *O/278*	10	*O/260 : O/274 : 260/278; 274/278*	10:12:7:9	1:1:1:1	1.38	0.7093
97-6							
cmrCg61	*216/216* × *216/216*	–	*216/216*	39	1	–	–
cmrCg141	*O/196* × *196′/178*	8	*(O, 196)/196′ : 196/178 : O/178*	23:6:10	2:1:1	2.27	0.3208
cmrCg143	*146/155* × *150/160*	12	*146/150 : 146′/160 : 150/155:155/160*	7:4:18:11	1:1:1:1	10.93	0.0121
cmrCg151	*281/281* × *281/281*	–	*281/281*	37	1	–	–

P_{cr} = 0.016, 0.025.

TABLE 7.

Analysis of selection at 12 microsatellite loci in 5 Pacific oyster families.

Family	*Type*	G_{Total}	P_{Total}	$G_{Parent1}$	$G_{Parent2}$	$G_{Gametic}$	$G_{Zygotic}$	$P_{Parent1}$	$P_{Parent2}$	$P_{Gametic}$	$P_{Zygotic}$	*Form*	*Action*
7 × 6													
ucdCg04	7	15.77	0.0004	–	–	1.11	14.65	–	–	0.2912	0.0001	Z	u.d.,s
ucdCg08	7	14.22	0.0008	–	–	2.67	11.55	–	–	0.1025	0.0007	Z	u.d.,s
ucdCg10	7	15.50	0.0004	–	–	4.76	10.74	–	–	0.0291	0.0010	Z	u.d.,s
ucdCg13	7	14.20	0.0008	–	–	14.19	0.01	–	–	0.0002	0.9278	G	d,p
93IL2													
ucdCg09	9	13.76	0.0033	1.14	6.31	7.45	6.31	0.2848	0.0120	0.0063	0.0120	G	d,f
ucdCg14	8	13.54	0.0012	–	–	2.79	10.74	–	–	0.0946	0.0010	G	d,m
ucdCg21	7	18.81	0.0001	–	–	16.91	1.91	–	–	<0.0001	0.1673	G	d,p
ucdCg28	9	15.96	0.0012	–	9.87	9.87	6.09	–	0.0017	0.0017	0.0136	G	d,f
97-2													
ucdCg06	9	39.72	<0.0001	37.16	1.33	38.49	1.23	<0.0001	0.2483	<0.0001	0.2669	G	m
ucdCg10	12	17.95	0.0004	10.20	0.14	10.34	7.61	0.0014	0.7054	0.0013	0.0058	G, Z	m,s
ucdCg13	8	21.87	<0.0001	–	–	20.22	1.65	–	–	<0.0001	0.1996	G	m
ucdCg15	8	12.11	0.0023	–	–	11.89	0.22	–	–	0.0006	0.6370	G	m
ucdCg143	9	14.92	0.0019	5.01	8.40	13.40	1.52	0.0253	0.0038	0.0003	0.2177	G	f
97-1													
cmrCg141	8	23.20	<0.0001	–	–	9.66	13.54	0.0019	–	0.0019	0.0002	G, Z	m,s
97-6													
cmrCg143	12	10.93	0.0121	8.40	2.53	10.92	0.01	0.0038	0.1119	0.0009	0.9271	G	m

G, allelic selection; Z, zygotic viability selection; u.d., underdominance; d, dominance; p, allelic selection by one of the parents, but unresolved; m, male allelic selection; f, female allelic selection; s, specific selection pattern.

Particular mention must be made of three loci: *ucdCg06*, *ucdCg08*, and *ucdCg10*. Locus *ucdCg06* is a four-allele system, with the sire being *156/182* and the dam *O/166*. Four progeny classes are expected, *156/O*, *156/166*, *O/182*, and *166/182*. This locus was examined in both young and old progeny and the *156* allele is weakly amplifying. In the first set of tests, of the juveniles, the *156* allele is detected twice. However, in the second set of (older) progeny, the *156* allele is not detected, although it is assumed to be present in progeny having the 166 or no amplification phenotype. Overall, there is significant distortion ($P < 0.0001$). It appears that this *156* allele is under strong selection in progeny due to a linked deleterious recessive gene or a deleterious interaction with factors transmitted by the dam.

For locus *ucdCg08*, the sire shows a single strong band at 249 bp and the dam shows a single strong band at 272 bp. However, the *249* allele is not detected, not even weakly, in any progeny. Progeny show either a 272-bp phenotype ($n = 15$), a 332-bp phenotype ($n = 10$; an allele that amplifies weakly), or no amplification product ($n = 7$). This locus is multiplexed with locus *ucdCg14*, which in this family presents four alleles where all individuals showed appropriate genotypes (no non-amplified individuals) and where the sire is consistent with the progeny genotypes given the dam. The progeny for *ucdCg08* are consistent with the following explanation: the dam is a *272/0* heterozygote and the sire is a *249/332* heterozygote. The absence of the *249* allele in progeny suggests that the *249* allele might mark a lethal interaction when combined with factors in the dam's genetic background (however, see "Discussion"). The *332* allele at *ucdCg08* does not amplify strongly using these PCR conditions (perhaps due to its larger size or a mutation in the priming site) and so is not reliably scored in the progeny; even though we can score the sire as a *249/332*, we score the borderline *332* allele as a null in progeny. The segregation at this locus fits expected Mendelian ratios when we score the dam's *272* allele as if it were a dominant segregation with the pooled class *272/-* and *O/O*. This will give two consistent phenotypic classes, 272, and null, in a 1:1 ratio—very close to the ratio observed.

Locus *ucdCg10* is a four-allele system, with the sire being *197/222* and the dam *153/175*. Four progeny classes are expected, *153/197*, *153/222*, *175/197*, and *175/222*. This locus was examined in both young and old progeny. In the first set of tests, of the spat, the 222-bp allele is below detection by the ABI system. However, in the second set of (older) progeny, the *222* allele is detected, although more weakly than the smaller alleles. Furthermore, while the segregation is close to expected in the spat, scoring the 222 as a null allele ($n = 32$, $P = 0.0451$), it is aberrant in the older individuals ($n = 31$, $P = 0.0003$), with overall significant distortion ($P = 0.0004$). This is due to a relative lack of the genotypes with weak *222* allele and a significant relative deficiency of the heterozygote *175/222* (Tables 5 and 7). We note that these four alleles are separated step-wise in size by about 20 to 25 bp—very close to the size of the forward PCR primer (Table 1). Whether changes in PCR kinetics (such as extension efficiency or priming errors) generated the variable detection of the *222* allele or whether there is an age-dependent effect was not resolved. Regardless of the amplification intensity of the *222* allele, it also appears that it is under selection in this family due to association with a deleterious gene, plus an interaction with the chromosomal segment marked by the *175* allele.

Fifteen of the 21 loci do not deviate significantly from Mendelian expectations. Of the six that deviate, three are borderline deviations from the adjusted significance levels. The three loci showing strong deviations were *ucdCg08* and *ucdCg10* (discussed above) and *ucdCg13*. For locus *ucdCg13*, 80.4% of progeny have the 148-bp phenotype instead of the 50% expected, apparently due to a linked deleterious effect associated with the 184-bp allele.

Family 4 (97-1 – A Non-Inbred Pair-Cross from Australia)

Four loci were tested (Table 6). One or other parent is heterozygous for a null allele and amplified allele in three instances. Three of the four loci accord with Mendelian expectations. The deviant locus, *cmrCg141*, gives progeny ratios that are quite different from those expected, even though all expected phenotypes are observed.

Family 5 (97-6 – A Non-Inbred Pair-Cross from Australia)

The same four loci were tested as in family 97-1 (Table 6). One parent is heterozygous for a null allele and amplified allele. Three of the four loci accord with Mendelian expectations and the fourth is marginally significant following corrections for multiple tests.

DISCUSSION

Null Alleles

A total of 47 tests of Mendelian transmission were carried out, 43 of which were segregating for more than one allele. Null alleles, including alleles such as $ucdCg10^{222}$ (see Table 5) with borderline amplification, were postulated in almost one-half of the tests (22 of 47 or 47%). It is possible that some borderline null alleles might amplify more intensely under less stringent PCR conditions (e.g. addition of more primer and lowering of annealing temperatures), but we have pooled these borderline nulls into the same category as completely null alleles. The completely undetectable null alleles might result from a more severe mutation (e.g. a small deletion in the priming site rather than an extended length of the microsatellite allele). Given that 43 informative segregation tests were carried out, $43 \times 4 = 172$ parental alleles were examined. Overall, 30 of the 172 parental alleles (17%) were null or non-amplifying alleles. One allele did not, for unknown reasons, amplify in a female parent ($ucdCg15^{S}$; Table 4). Null allele counts were slightly stock-dependent, being more common in Australian stocks (22 of 100 parental alleles, 22%) than North American stocks (8 of 72, 11%), however the difference is only marginally significant ($P = 0.04$). More crosses have to be examined to comment meaningfully on null allele incidence at individual loci, although there is a suggestion that null alleles might be more common at some loci than others. For example, *ucdCg04* was examined in three crosses (two North American and one Australian) with no evidence for null alleles, while at the other extreme, *ucdCg09* was examined in two crosses (one North American and one Australian) and in both cases the apparently homozygous parents were heterozygous for amplified and null alleles.

The presence of null alleles is often presumed to result from the primer design process or PCR artifacts such as paralogous loci, but progeny tests reveal that null alleles are inherited and result from mutations in the template. Multiplex PCR allows us to rule out non-specific inhibitors of PCR or poor template preparations as explanations of null alleles. Sequence specific inhibitors cannot be ruled out. The presence of null alleles in multiplex PCR reactions in one family and not another rules out systematic mismatches of the primers and implicates either template variation at the priming site or perhaps large insertion events that prevent extension during the thermal cycling. The high frequency of null alleles is not solely a property of any one genomic library or microsatellite primer set used. To date, multiple independent libraries have produced primers with null alleles in Pacific oysters (McGoldrick 1997, Magoulas et al. 1998, English unpublished). Multiple laboratories have observed null alleles in oysters, some running other types of PCR markers. For example, Hu and Foltz (1996), while examining scDNA markers in American oysters, found three instances of "abberant genotypes" in families that can be explained by null alleles.

Since null alleles occur in one pedigree and not others, primer design is rather "hit and miss." Redesigning primers is feasible if a few loci (perhaps five) are to be typed for population genetic work or for a limited number of pedigrees, but becomes much more difficult for a bivalve genome mapping study requiring about 100 markers as anchor loci and with complete codominant expression for all alleles. There is no *a priori* mechanism to guarantee that nulls will not appear at the redesigned primers in a new pedigree or in some proportion of priming sites in a natural population. Nevertheless, it is quite clear from these breeding studies that null alleles are frequent at microsatellite loci in Pacific oysters and perhaps all PCR-based markers in bivalves that amplify neutral DNA.

Null alleles at allozyme loci have been reported in oysters. In the American oyster (*C. virginica*) null alleles were reported at *Mpi* and *Lap-2* after testing progeny from five crosses (Foltz 1986b). The importance of these relatively rare observations at allozyme loci in influencing general deficiencies in heterozygosity has been down-weighted relative to larval viability selection (e.g. Mallet et al. 1985, Gardner 1992). Overall, heterozygote deficiencies for microsatellite loci in Tasmanian and Japanese samples Pacific oysters (English unpublished, McGoldrick and Huvet unpublished) appear to be considerably larger than those observed for allozyme loci (English et al. 2000). This is most likely related to the higher prevalence of null alleles for microsatellites than for allozymes, and suggests there is a greater level of segregating variation in untranscribed DNA than in transcribed DNA. Sequence evolution at priming sites can be explained by additional mutational mechanisms, perhaps involving recombination, that act in addition to neutral point mutations (e.g. through deletions and insertions) and with longer persistence in neutral DNA. Alternatively, population level phenomena such as hybridization and introgression of diverged chromosomal segments (Hirase 1930, Imai and Sakai 1960, Thomson 1959, also reviewed by Gardner 1997) might also be important. Hybridization and hypotheses about differences between transcribed and untranscribed DNA have some published basis and the relative contribution of each remains unresolved at this time.

Null Alleles in Gene Mapping

Nulls can be accommodated in gene mapping. Given any microsatellite segregation, the most complete classification would be parents "*A/B* × *C/D*" crossed to give progeny classes "{*A/C, A/D, B/C, B/D*}" (type 12 in Table 2). Should one of these alleles be a null allele, the segregation would be scored in the form "*A/C, A/O, B/C, B/O*" (e.g. type 10). Note that the change in score from observed allele *D* to unobserved allele *O* does not change the underlaying counts in these progeny categories, the segregation tests, degrees of freedom, or the mapping results. Therefore, changing the priming sites or conditions to favor amplification of the *D* allele does not produce any statistical gain in mapping. Further, should the allele *C* in the second parent be indistinguishable from the *A* or *B* allele in the other parent (reducing the cross type from "*A/B* × *C/O*" to "*A/B* × *B/O*"; type 5), the segregation would be of the form "*A/B, B/B, B/O, A/O*". This would be equal

in information content to the standard segregation of "*A/B* × *B/B*". Here we simply pool the classes "*A-*" and "*B-*" recognizing the "*A/O*" as a legitimate genotypic class and achieve equal power to the "*A/B* × *B/B*" case. Similarly, should both alleles in one parent be null and the other parent heterozygous amplified/null (type 2), the results are again equal to an "*A/B* × *B/B*" segregation. Moreover, should one parent be heterozygous for a null and the other homozygous for nulls (type 0), we again have equal power to the "*A/B* × *B/B*" segregation if template reactions are controlled with multiplex PCR so that the "*O/O*" genotype can be scored reliably. If both parents are heterozygous for nulls and indistinguishable amplified alleles "*A/O* × *A/O*" (or type 1), we would have some mapping information (equivalent to a 3:1 segregation), but would not have expected any in the case of "*A/A* × *A/A*". Lastly, we note that if both parents are heterozygous for nulls and distinguishable amplified alleles (type 9), we have four recognizable classes and complete classification, e.g. "*A/O* × *B/O*" gives "*A/B, A/O, B/O, O/O.*"

These facts suggest that at worst we get a 1:1 ratio, and in large studies, decreasing gain for effort in resolving null issues by reducing PCR stringency or redesigning primers for highly variable markers. Therefore, should a mapping project with 50 to 100 microsatellites plan for this level of power (e.g. using dominant AFLP markers and microsatellites together), there is little problem with null alleles in mapping. Certainly, the great fecundity of bivalves can accommodate the 398 progeny needed to detect loose (30 cM) linkages in the assortment of two dominant loci (see Allard [1956]) for a detailed analysis of the numbers of progeny needed for linkage mapping with dominant markers). Indeed, organisms with large family sizes (e.g. most invertebrates and plants), even if they have null alleles, have obvious natural advantages that make them desirable for mapping with microsatellites.

Selection Associated with Microsatellite Markers

About one-third of microsatellite segregation ratios showed significant ratio distortion (16 of 43 or 37.2%) when null alleles are accounted for. This proportion comes after significance adjustments are made within each of the five crosses for multiple tests of segregation. There is no significant association between segregation distortion and the presence of null alleles (12 tests showed no distortion and null alleles, 15 showed no distortion and no null allele, eight showed distortion and null alleles, and eight showed distortion and no null allele, $P = 0.724$).

Segregation distortion at the level of genetic markers is clearly quite common in Pacific oysters. Allozyme analysis of the progeny of sibs from selfed hermaphrodite Pacific oysters also revealed significant segregation distortion (McGoldrick and Hedgecock 1997), again affecting about one-third of tests (16 out of 51 or 31%). Significant distortion has also been recorded in the flat oyster, *Ostrea edulis*, again in about one-third of tests of microsatellite segregation (Naciri et al. 1995, Bierne et al. 1998). Many other published studies, most of which are based on allozyme data (Appendix 1), have also reported segregation distortion in bivalves. Thus the distortion does not appear to be associated with the use of microsatellite loci, but is a general bivalve phenomenon. In our studies, strong zygotic viability selection cannot be ruled out.

Selection in Bivalves

There is much to be gained by investigation of the forms of selection and markers associated with segregation distortion in other bivalves (Appendix 1). Selection is often associated with single alleles (also sometimes referred to as "gametic" whereby selection can be explained by simple deleterious recessive genes linked to an observed allele). For simple segregation ratios of type 0 through 6 (Table 2), apparent selection on specific genotypes has been reported in the genus *Crassostrea* 19 times (*C. virginica* 16 times and *C. gigas* three times). No data were found for flat oysters (genus *Ostrea*), but in Mytilidae (represented by *Mytilus edulis*), distortion has been reported five times for these seven types. The simpler types of segregation (type 0–6) cannot ever reveal any underlying non-additive properties to the selection observed at the level of these markers, so the context within which the observation is made can lead to a biased conclusion which implicates only linked deleterious recessives. In fact, deleterious recessive action alone would grossly under-represent gene action at genetic markers in bivalves. This can be observed when types of crosses and markers are used that have more informative segregation (e.g., types 7–12).

For more informative segregation ratios, selection on specific genotypes apparently occurred in all four taxa (*C. gigas*, *C. virginica*, *O. edulis*, and *M. edulis*). There were 14 such segregation distortion events in the American oyster (*C. virginica*), one case outside this study in the Pacific oyster (*C. gigas*), 18 cases in mussels (*M. edulis*), and three cases in flat oysters (*O. edulis*; Appendix 1). No attempt is made to correct for experimental effort in these cases, but by taking these events case by case and when the information is available, underdominant selection patterns often appear (with a lower fitness for the heterozygote). This implies there are associated negative interactions between chromosomal segments containing the observed locus. For example, in the dihybrid crosses with distortion (type 7 crosses), five of seven events (71%) included some form of underdominance and this occurred in all four bivalve taxa (Appendix 1). The present study concurs. For example, the North American family 7 × 6 has three of four cases (75%) apparently underdominant using the microsatellite markers (Tables 3 and 7). These results are intriguing when combined with the empirical observation at locus *ucdCg08* concerning the 249-bp allele (Table 5). The absence of the *249* allele in progeny suggests that the *249* allele might mark a lethal interaction when combined with factors in the dam's genetic background. An alternative hypothesis is that the sire's *249* allele is a paralogous amplification product not belonging to *ucdCg08*, but this would not explain why the 249-bp fragment was not observed in any progeny nor why it had a microsatellite stutter pattern. In addition, if there was some sort of template contamination in the sire that produced the *249* allele, then all the other loci in this family should have had spurious alleles observed in the sire, but no unexplained alleles were observed at other loci. While the segregation of the 249 allele might be an unexplained artifact, it is also consistent with an interaction hypothesis.

This underdominance at the family level is potentially very serious because gene frequencies become naturally unstable with major population genetic and evolutionary implications in terms of frequency dependent selection. Underdominance in segments (including all sufficiently linked genes) can lead to fixation of introgressed gene segments in metapopulations or randomly drifting small populations of bivalves should one or the other segment attain and maintain a critical frequency threshold (e.g. Crow 1986). This is particularly relevant in light of low estimated effective sizes in hatchery stocks (Hedgecock and Sly 1990). Further, it is possible to cause extinction by means of gene replacement if an introgressed segment confers susceptibility to some periodic environmental trigger such as a disease outbreak or environmental shift. The potential cost of this type of event could be the value of

the entire culture industry if a commercial broodstock becomes affected and is not managed with this contingency in mind. In evolutionary terms, bivalve populations might remain well adapted and genetically stable for long periods of time, but might unexpectedly and rapidly approach extinction in response to a recurrent environmental trigger. Further research into the prevalence of underdominance in bivalves is needed and awaits more defined linkage mapping especially within the context of stock importation and gene frequency modification during selective breeding.

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APPENDIX 1.

Reported occurrences of segregation distortion for bivalve Mollusc families.

Species	Cross	*Locus*	*Parent 1 × Parent 2*	*g1*	*g2*	*g3*	*g4*	*Ratio*	H_0	*G*	*P*
Type 3 AB × AA											
C. virginica	Cross 1	*Ap-1*	*100/108'*	*108/108*	*108'/108*	*108/100*		18:44	1:1	11.25	0.0008[c]
C. virginica	Cross 1	*Gpi*	*100/100*	*78/58*	*100/78*	*100/58*		18.44	1:1	11.25	0.0008[c]
C. virginica	Cross 2	*6PGDH*^{ale}	*100'/72*	*100/100*	*100/100'*	*100/72*		318:157	1:1	55.67	0.0000[c]
C. virginica	Cross 4	*Pgm-2*	*100'/82*	*100/100*	*100'/100*	*100/82*		38:9	1:1	19.25	0.0000[c]
C. virginica	Cross 5	*Pgm-2*	*100/82*	*100/100*	*100/100*	*100/82*		412:299	1:1	18.04	0.0000[c]
C. virginica	Cross 7	*Lap-1*	*100/93*	*100/100*	*100/100*	*100/93*		209:170	1:1	4.02	0.0450[c]
C. virginica	Cross 7	*Lap-2*	*100/100*	*100'/83*	*100/100'*	*100/83*		109:266	1:1	67.80	0.0000[c]
C. virginica	Cross 10	*Got-2*	*78/100'*	*100/100*	*78/100*	*100/100'*		25:8	1:1	9.19	0.0024[c]
C. virginica	Cross 10	*Lap-2*	*100/83*	*100/100*	*100/100*	*100/83*		25:8	1:1	9.19	0.0024[c]
C. virginica	Cross 10	*Gpi*	*e/c*	*c/c*	*e/c*	*c/c*		32:14	1:1	7.24	0.0071[d]
C. virginica	Cross 10	*Gpi*	*e/c*	*c/c*	*e/c*	*c/c*		61:36	1:1	6.52	0.0107[d]
C. virginica	Cross 4	*CV-195*^{Agalt}	*B/B*	*A/B*	*(A/A)*	*A/B*	*B/B*	5:3:24	0:1:1	0.00	0.0000[e]
C. gigas	92-97-5	*Pgm*	*A/B*	*A/A*	*A/A*	*A/B*		9:24	1:1	7.07	0.0078[f]
C. gigas	92-97-5	*Lap-2*	*A/C*	*C/C*	*A/C*	*C/C*		9:24	1:1	7.07	0.0078[f]
C. gigas	92-97-5	*Dap-2*	*A/A*	*A/B*	*A/A*	*A/B*		21:6	1:1	8.83	0.0030[f]
M. edulis	FAM II	*Est-D*	*100/121*	*100/100*	*100/100*	*121/100*		85:26	1:1	33.03	0.0000[a]
M. edulis	FAM II	*Pgd*	*3/4*	*3/3*	*3/3*	*4/3*		109:62	1:1	13.09	0.0003[a]
M. edulis	64 × 5	*Pgi*	*100/100*	*88/100'*	*100/100'*	*88/100*		96:126	1:1	4.07	0.0437[b]
M. edulis	29 × 10	*Lap*	*100/100*	*100'/105*	*100/100'*	*100/105*		16:34	1:1	6.63	0.0100[b]
M. edulis	64 × 101	*Lap*	*100/105*	*100/100*	*100/105*	*100/100*		78:131	1:1	4.80	0.0285[b]
Type 4 AB × CC											
C. virginica	Cross 7	*Gpi*	*112/78*	*100/100*	*112/100*	*100/78*		220:162	1:1	8.84	0.0029[c]
C. virginica	Cross 8	*Gpi*	*100/100*	*112/78*	*112/100*	*100/78*		113:75	1:1	7.73	0.0054[c]
M. edulis	64 × 101	*Pgi*	*100/100*	*90/96*	*96/100*	*90/100*		107:148	1:1	6.62	0.0101[b]
Type 6 AA × BC											
C. virginica	Cross 4	*Got-2*	*100/100*	*78/279*	*78/100*	*100/279*		14:31	1:1	6.58	0.0103[c]
C. virginica	Cross 5	*Got-2*	*100/100*	*78/279*	*78/100*	*100/279*		291:411	1:1	20.61	0.0000[c]
C. virginica	Cross 5	*Lap-2*	*115/115*	*100/00*	*115/00*	*115/100*		413:335	1:1	8.15	0.0043[c]
C. virginica	Cross 5	*Gpi*	*100/100*	*78/58*	*100/78*	*100/58*		314:440	1:1	21.15	0.0000[c]
Type 7 AB × AB											
C. virginica	Cross 3	*Mpi*	*91/100*	*91/100*	*91/91*	*(91/100)*	*100/100*	55:88:70	1:2:1	8.26	0.0160[c]
C. virginica	Cross 10	*Gpi*	*e/c*	*e/c*	*(e/e)*	*e/c*	*(c/c)*	14:68:11	1:2:1	21.02	0.0000[d]
C. gigas	92-97-5	*Acon-1*	*A/C*	*A/C*	*A/A*	*(A/C)*	*C/C*	21:5:1	1:2:1	33.92	0.0000[f]
M. edulis	FAM I	*Est-D*	*100/121*	*100/121*	*100.100*	*(121/100)*	*121/121*	73:90:22	1:2:1	29.01	0.0000[a]
M. edulis	64 × 5	*Lap*	*100/105*	*100/105*	*105/105*	*(100/105)*	*100/100*	43:75:102	1:2:1	47.38	0.0000[b]
M. edulis	64 × 5	*Lap*	*100/105*	*100/105*	*105/105*	*(100/105)*	*100/100*	43:75:102	1:2:1	47.38	0.0000[b]
O. edulis	C1	*Oedu.J12*^{70}	*224/230*	*224/230*	*224/224*	*(224/230)*	*230/230*	17:9:52	1:2:1	70.95	0.0000[g]
O. edulis	C2	*Oedu.B0*^{70}	*98/101*	*98/101*	*98/98*	*98/101*	*(101/101)*	25:50:5	1:2:1	19.61	0.0001[g]
Type 8 AB × AO											
C. virginica	Cross 2	*6PGDH*	*100/72*	*100'/O*	*100/–*	*100'/72*	*(O/72)*	318:157:0	2:1:1	0.02	0.8967[c]
C. virginica	Cross 4	*CV-195*^{Agalt}	*B/O*	*A/B*	*(A/O)*	*(A/B)*	*B/–*	5:3:24	1:1:2	8.88	0.0118[c]
Type 11 AB × AC											
C. virginica	Cross 2	*Got-2*	*78/100*	*78'/279*	*78/78'*	*78'/100*	*78/279*	*100/279*	81:107:101:172	1:1:1:1	38.05	0.0000[c]
C. virginica	Cross 3	*Got-2*	*78/100*	*78'/279*	*78/78'*	*(78'/100)*	*78/279*	*100/279*	51:22:66:69	1:1:1:1	30.68	0.0000[c]
C. virginica	Cross 3	*Gpi*	*100/78*	*78'/58*	*100/78'*	*100/58*	*78/78'*	*78/58*	27:72:48:73	1:1:1:1	28.63	0.0000[c]
C. virginica	Cross 4	*Pgm-2*^{ale}	*100'/82*	*100/O*	*100/–*	*100/82*	*(O/82)*	38:9	2:1	4.68	0.0305[c]
C. virginica	Cross 8	*Ap-1*	*116/108*	*116'/100*	*(116/116')*	*116'/108*	*116/100*	*(108/100)*	37:57:58:33	1:1:1:1	11.29	0.0102[c]
C. virginica	Cross 10	*Lap-1*	*100/82*	*100'/93*	*100/100'*	*100/93*	*100'/82*	*93/82*	11:0:16:12	1:1:1:1	23.49	0.0000[c]
M. edulis	FAM I	*Pgm-2*	*100/92*	*100/80*	*100/100*	*100/92*	*100/80*	*92/80*	46:30:35:23	1:1:1:1	8.32	0.0398[a]
M. edulis	FAM I	*Hex*	*100/157*	*100/28*	*100/28*	*157/28*	*100/100*	*157/100*	73:13:69:23	1:1:1:1	70.44	0.0000[a]
M. edulis	FAM II	*Lap*	*94/96*	*94'/98*	*94/94'*	*96/94'*	*98/94*	*98/96*	27:50:32:49	1:1:1:1	10.67	0.0136[a]
M. edulis	32 × 10	*Pgi*	*93/100*	*88/93*	*93'/100*	*88/100*	*93/93'*	*88/93*	76:29:16:8	1:1:1:1	79.40	0.0000[b]
M. edulis	45 × 39	*Pgi*	*91/100*	*93/100'*	*100/100'*	*93/100*	*91/100'*	*91/93*	63:101:59:86	1:1:1:1	15.11	0.0017[b]
M. edulis	45 × 42	*Pgm*	*100/103*	*98/103'*	*103/103'*	*100/103'*	*98/103*	*98/100*	13:35:14:23	1:1:1:1	14.11	0.0028[b]
M. edulis	29 × 10	*Pgm*	*97/100*	*100'/107*	*100/107*	*97/107*	*100/100'*	*97/100'*	36:71:55:74	1:1:1:1	16.52	0.0009[b]
M. edulis	32 × 10	*Pgm*	*94/107*	*100/107'*	*107/107'*	*100/107*	*94/107'*	*94/100*	47:34:29:19	1:1:1:1	12.73	0.0053[b]
M. edulis	64 × 5	*Pgm*	*97/100*	*100'/104*	*100/104*	*97/104*	*100/100'*	*97/100'*	47:32:67:50	1:1:1:1	12.76	0.0052[b]
M. edulis	64 × 101	*Pgm*	*97/100*	*97'/104*	*100/104*	*97/104*	*97'/100*	*97/97'*	30:39:74:67	1:1:1:1	26.72	0.000[b]
O. edulis	C1	*Oedu.T5*^{10}	*106/124*	*124'/128*	*124/124'*	*124/128*	*106/124'*	*106/128*	17:12:32:25	1:1:1:1	11.01	0.0117[g]
Type 12 AB × CD											
C. virginica	Cross 1	*Lap-2*	*115/108*	*100/00*	*115/00*	*115/100*	*108/00*	*108/100*	15:8:24:7	1:1:1:1	13.21	0.0042[c]
C. virginica	Cross 2	*Gpi*	*112/100*	*78/58*	*112/78*	*112/58*	*100/78*	*100/58*	65:109:109:194	1:1:1:1	70.74	0.0000[c]
C. virginica	Cross 4	*Gpi*	*106/100*	*78/58*	*106/78*	*106/58*	*100/78*	*100/58*	9:21:4:14	1:1:1:1	13.85	0.0031[c]
C. virginica	Cross 8	*Adk*	*110/92*	*100/96*	*110/100*	*110/96*	*100/92*	*96/92*	38:57:28:36	1:1:1:1	10.91	0.0122[c]

[a] Beaumont 1983. [b] Hvilsom and Theisen 1984. [c] Foltz 1986. [d] Hu et al. 1993. [e] Hu and Foltz 1996. [f] McGoldrick 1997. [g] Bierne 1998.

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MICROGEOGRAPHIC DIFFERENCES IN GROWTH, MORTALITY, AND BIOCHEMICAL COMPOSITION OF CULTURED PACIFIC OYSTERS (*CRASSOSTREA GIGAS*) FROM SAN QUINTIN BAY, MEXICO

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ABSTRACT Changes in shell height, tissue dry weight (TDW), mortality, and biochemical composition of *Crassostrea gigas* were evaluated at two commercial sites in San Quentin Bay (SQB), Mexico, during the first 9 mo post-settlement. Shell growth rates were 1.5× higher at the mouth (0.36 mm d^{-1}) than the head of SQB (0.23 mm d^{-1}). Tissue dry weight was also 7- to 8-fold higher in oysters from the mouth (2.1–2.16 g TDW $oyster^{-1}$) than the head of SQB (0.23–0.33 g TDW $oyster^{-1}$) at the end of the 9-mo experiment. Market size (9 cm) was reached after 9 mo at the mouth and at an estimated age of 13 mo at the head of the bay. Highest mortality occurred within the first month post-settlement (50%–60%) and reached approximately 63% to 87% throughout the whole study period. Proteins (48%–64%) and lipids (2%–9%) were the most abundant biochemical components during early spat development. When oysters reached a size between 48 and 55 mm (shell height) in the fall, glycogen (1%–22%) replaced lipids as the main energy depot. The changeover occurred earlier in oysters at the mouth than in oysters from the head of the bay. It is suggested that between-site differences in growth and biochemical composition in oysters are the result of longer immersion/feeding period experienced by oysters at the mouth of SQB.

KEY WORDS: Oyster, *Crassostrea gigas*, San Quentin, biochemical content, growth, condition index

INTRODUCTION

San Quentin Bay (SQB) is a shallow coastal lagoon (2 m mean depth) located between 30° 24′ and 30° 30′N and 115° 57′ and 116° 01′W, on the northwest Pacific coast of Mexico. Its productivity (0.24–0.94 g C m^3 d^{-1}) and hydrodynamics are strongly tide-dependent and influenced by alongshore upwelling systems, which maintain a high nutrient supply to the lagoon during the spring months (Alvarez-Borrego and Alvarez-Borrego 1982, Millan-Nuñez et al. 1982). The residence time of the water in the bay varies from hours near the mouth to days at the head of SQB (Juarez-Villarreal 1982). Therefore, any changes in the available food, either in quantity or quality, or any microgeographic differences in environmental conditions may have implications for the successful culture of suspension-feeders.

Hatchery production of commercially important marine bivalves, such as oysters, is well established and provides the basis for economically viable industries on both coasts of North America. The Pacific oyster, *Crassostrea gigas*, is a species introduced from Japan and it is the mainstay of the oyster industry in the Pacific coast. This industry relies largely on hatchery production of larval stages in American hatcheries and remote setting of pediveligers around the world. *C. gigas* was introduced into SQB (Ensenada, Mexico) in 1975, and its acceptance in the local market prompted the adaptation of mass production techniques in the laboratory and the field by local farmers (Islas-Olivares 1975). While larval cultures proved to be uneconomical, oyster production via remote setting increased from approximately 100 to 2,000 tons live weight $year^{-1}$ over the last 20 years (J.C. Garduño pers. comm.). This production was based on remote setting of pediveliger larvae on oyster or scallop shells, early spat rearing in subtidal rafts, and suspended juvenile-adult grow-out in intertidal structures called "racks."

The substrate with newly settled oyster spat is typically placed inside wide-mesh bags and suspended from floating rafts along the channels of SQB. At the end of the rearing period, a series of 7 mother shells are connected through a polypropylene rope (overall length 1.2 m) and suspended in intertidal culture "racks" made of wood or PVC plastic frames (Polanco et al. 1988). The oysters remain in these units (approximately 6.2 × 2 × 1.2, length × width × height) until they reach the harvesting size of approximately 9 to 10 cm (Polanco et al. 1988). The culture process is extensive, since most operations involve a substantial amount of human labor and minimal manipulation once the oysters are transferred to the water column. The efficiency of these commercial units remain anecdotal, largely because the lack of coordination between aquaculturists and researchers, and the budget constraints to travel to and from SQB, a pristine site located approximately 250 km from academic centers. A previous study (Acosta-Ruiz 1985) reported oyster growth rates on experimental systems different from those commercially utilized. Therefore, the present study was aimed at assessing the growth, mortality, and biochemical changes of the Pacific oyster, *C. gigas* during two production cycles at two commercial sites of SQB with differing residence water times.

MATERIALS AND METHODS

Setting and Early Rearing

Two batches of oyster larvae were sent overnight inside insulated boxes in the form of a wet "paste" wrapped with nylon cloth and paper towels. Ice packs were also placed inside the boxes in order to maintain a cold atmosphere (approximately 5 °C) during transport. Full details for the storing and shipping conditions of competent (ready-to-set) larvae are given elsewhere (Jones and Jones 1983). Both oyster batches came from the Whiskey Creek

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TABLE 1.

Morphometry and settlement success of two oyster (*Crassostrea gigas*) batches in San Quintin Bay. Spring and Summer = larvae were set in April and July of 1995, respectively. Mean ($\bar{X}$ = ±95% confidence interval) or median values (Md, was 25% and 75% percentile range) are shown for each variable. Mann-Whitney test was used for comparison of medians. Comparisons of means were carried out with a two-sample Student test. ns = Non-significant difference, ** = Significant differences at $\alpha = 0.01$, NS.

Variable	Spring batch	Summer batch
Shell height (μm)	Md = 321.8 (316.8–326.7)	Md = 236.7 (316.8–326.7); ns
Dry weight (μg)	Md = 6.6 (6.1–7.0)	Md = 5.5 (5.4–5.7); **
Ash-free dry weight (μg)	$\bar{X}$ = 2.20 (±0.06)	$\bar{X}$ = 2.14 (±0.04); ns
Shell width (μm)	$\bar{X}$ = 297.6 (±2.7)	$\bar{X}$ = 290.2 (±2.7); **
Setting		
No. shells per tank	63,000	30,000
No. larvae per milliliter	1.2	0.6
Setting time	59 h	48 h
No. spat per shell	$\bar{X}$ = 46.5 (±7.4)	$\bar{X}$ = 29.9 (±4.5)

Oyster Farm (Oregon) on April 20 (Spring experiment) and July 20, 1995 (Summer experiment). Upon arrival to the Instituto de Investigaciones Oceanológicas in Ensenada (I.I.O.), triplicate subsamples of 30 to 100 larvae were individually measured under a microscope and weighed in terms of dry and ash-free dry weight (see below). The rest of the larval paste was packed again as described above, and transported by land (approximately 4 h) to SQB where oyster setting took place. Overall shipping time from Oregon to SQB was approximately 36 h.

Larval setting was fully carried out by local fisherman. Briefly, about 400 half-oyster shells were packed in individual bags made of nylon rope (diagonal mesh 7 cm). Bags with shell substrate were deployed in cylindrical fiberglass tanks of 8,500 L (diameter = 300 cm, height = 120 cm). The total amount of bags with shell substrate per setting tank was different for the (Spring) and (Summer) batches (Table 1), but the ratio of larvae:substrate was about the same in both cases, e.g. 170:1 (swimming larvae:shell). These numbers are about twice as much the ratio typically used by oyster growers in America (Jones and Jones 1983), but no attempt was made to modify local practices. Setting took place over 60 h (Spring) or 48 h (Summer) under closed conditions and aeration was provided through air stones. At the end of the setting period the bags with substrate were transferred to subtidal floating rafts located along the channels of SQB (Fig. 1), where they remained until ready for grow-out, in intertidal racks.

Mortality

Prior to transferring the newly settled spat to the rearing channels, a total of 21 (Spring) and 33 (Summer) shells were removed from the bags and labeled. The number of spat attached to the inner side of the shell was counted with the aid of magnifying glasses and recorded. The same shells were recovered at the end of the rearing period in order to count the number of spat present in their internal and external sides. Percentage of mortality during the rearing phase (floating rafts) was based on the number of spat set on the internal side of the shell. From this point on both sides of the shell were used for mortality estimates.

At the end of the rearing period, the shells with spat were strung on sections (1.2 m length) of polypropylene rope. The entire assembly is locally known as "sarta," and consists of 7 equally spaced shells per rope. Ten sartas with shells were labeled and suspended in intertidal culture racks located at the head (Fig. 1) and near the mouth of SQB (5 sartas per site, 1 sarta per rack). All experimental sartas were intermingled with the rest of those deployed by local fishermen (total 110 sartas per rack) in order to avoid any density-dependent bias throughout the experiments. In the Spring experiment, monthly/bimonthly mortality was assessed "*in situ*" by counting the total number of live and dead oysters in each sarta. Evaluations for the Summer experiment were carried out at the beginning of the experiment (post-settlement), the end of the rearing phase (or beginning of grow-out phase), and the end of the experiment. Empty shells (open valves and no tissue) were recorded as dead organisms in all cases. At the end of the experiment, all 10 sartas were taken to the laboratory where the oysters were carefully detached from their substrate and counted. The percentage of cumulative mortality (%M) was calculated by using the following equation:

$$\%M = [1 - (N_t/N_0)] \times 100$$

where N_0 = initial number of live oysters per sarta and N_t = number of live oysters at time t.

Growth and Biochemical Changes

At the same sites selected for mortality evaluations (Fig. 1), 120 sartas were labeled and suspended in 10 commercial culture

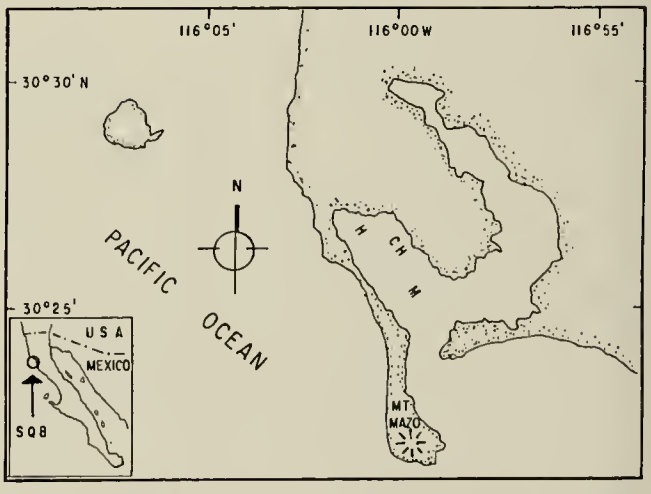

Figure 1. Rearing and grow-out experimental sites in SQB. *C. gigas* was reared subtidally at the channel (CH) during the first month of post-larval life. Further grow-out was carried out at the head (H) and near the mouth (M) of the left of SQB.

racks. A total of 5 racks per site and 12 sartas per rack were used for this experiment. Between 3 and 10 sartas (1 per culture rack) were monthly/bimonthly collected from each site, taken to the laboratory inside coolers, and placed in a cold room (4 °C) until processing the next day.

Handling of larvae, sarta's manufacturing, and grow-out operations were carried out by local fishermen. Labeling, distribution of experimental sartas, sampling, and processing were carried out by us in order to avoid any bias in estimating the actual efficiency of the production units.

Sample Processing

Setting larvae were placed in pre-combusted aluminum pans and rinsed with a small amount (approximately 1 mL) of isotonic ammonium formate (2.75%, w/v) to eliminate salts. The solution was quickly removed from the pans with the aid of a Pasteur pipet and tissue paper. Pans with larvae were oven-dried at 60 °C for 24 h, weighed, and combusted at 450 °C for 24 h. The ash-free dry weight of oyster spat was obtained by the difference between dry and ash weights.

The sartas with oysters were processed in the laboratory as follows: oysters were removed from the substrate with the aid of a flat-head screwdriver and hammer. The shell substrate had to be carefully broken in order to separate intact oysters located on the inner side of the substrate or hidden between other experimental oysters, and therefore the use of a screwdriver was preferred instead of a knife. Fifteen intact oysters per sarta were randomly selected and measured with digital calipers (±0.01 mm) on their longest dimension and weighed (live weight). The shell and soft tissue of each individual were then separated in order to estimate condition index. The shell was dried at room temperature for 24 h and weighed, while the whole tissues were oven-dried (105 °C, 24 h) and weighed. The remaining oysters were frozen at −20 °C and freeze-dried for later biochemical analysis.

Condition index (CI) was calculated on a gravimetric basis using the following equation (Crosby and Gale, 1990):

$$CI = [\text{tissue dry weight (g)} \times 1{,}000]/[\text{capacity of the inner side of the shell}]$$

where the capacity of the inner side of the shell (g) is the difference between the weight of whole (live) oyster and the dry weight of the shell.

Tissue weight of small (<3 cm) oyster spat was determined by carefully separating their shells with the aid of dissecting forceps under microscope, followed by complete tissue/fluid recovery with Pasteur pipettes, transfer to aluminum pans, and oven-drying at 105 °C for 24 h. No attempt was made to estimate the condition index of these small oysters, due to the impossibility of separating intact organisms from the shell substrate.

Weighing of larvae/early oyster spat was done on a Perkin-Elmer AD2Z electrobalance (±0.001 mg) while juvenile/adult stages were weighed on a Mettler balance (±0.1 mg). Absolute growth rates (AGR, μm d^{-1}) were computed from linear regressions from the slope obtained between shell height and age of the oysters.

Biochemical analyses were performed on Spring oysters only. Six to 35 oysters per sarta (depending on size) were dissected, and their adductor muscle and remaining tissues (viscera, gills, and mantle) were pooled into two separate fractions. Three replicates (sartas) were processed on each sampling date, but pooled sartas were used when needed for small sizes. Muscle and remains were freeze-dried, pulverized with the aid of a coffee grinder, and stored (−70 °C) inside tightly closed jars containing dessicant. These samples were later used for biochemical analyses in 1997. Total tissue nitrogen (N) was quantified with the micro Kjheldahl method (AOAC 1990) and proteins were estimated by multiplying N × 5.8 (Gnaiger, 1983). Tissue glycogen was precipitated with ethanol, converted to glucose by acid hydrolysis, and quantified enzymatically (Pfleiderer 1983). Lipids were extracted with ethanol:chloroform (2:1 and 1:2, Bligh and Dyer 1959) and quantified gravimetrically.

Environmental Variables

One thermograph (Ryan Instruments, mod. TempMentor) was deployed at the mouth of SQB on July 10, 1995. Another termograph (I.I.O.-UABC, mod. TDS-85) was also deployed at the head of SQB on the same date and continuous (hourly) temperature recordings were obtained through November 14, 1995.

Monthly evaluations of total particulate matter (TPM) were carried out for each location between July 1995 and April 1996. Triplicate water samples were collected in plastic bottles (250 mL) at 65 cm above bottom with the aid of manual vacuum pumps (approximately 25 cm Hg, vacuum). A nitex screen (30-μm mesh) was placed at the entrance of the tubing in order to remove larger particles. The samples were transported to the university inside coolers (approximately 2°C–4 °C) with a total elapsed time between collection and processing of less than 8 h. Water samples were filtered through pre-washed and pre-combusted glass fiber filters (GF/F). A final rinse with isotonic ammonium formate (2.75%) was done in order to eliminate salts. The filters were dried at 90 °C for 20 h, weighed, combusted at 450 °C for 2 h, and re-weighed. TPM was obtained directly from the dry weight and the organic fraction (POM) was obtained by the difference between the ash and dry weights.

Statistics

A multifactorial two-way ANOVA (2 × 2 × 5) was used to test the effects of the seeding season (Spring and Summer) and culture site (mouth and head) on the mean observed mortality of oysters at the end of the experiment, with 5 replicates (sartas) per site. Absolute growth rates of oysters cultured on different sites and seeding seasons were compared with a test of multiple comparisons of slopes (*a posteriori* Tukey's test). The same test was used to compare tissue growth, previous transformation of raw data (mg dry weight) to Ln (1 + weight in grams). A two-sample parametric (Student *t*) or non-parametric test (Mann-Whitney) was used at each experimental site to test the effect of seeding season (Summer versus Spring) on the oysters' condition index. Time effects were not statistically tested because the effect of this factor was obvious in all cases. A two-way ANOVA test was independently used to compare the effects of time (age) and tissue type (muscle and viscera) on the relative (percentage) protein, lipid, and glycogen content in oysters from the mouth and head of SQB.

With the exception of the comparisons of slopes (Zar 1984), the rest of the statistical tests were carried out on a personal computer with the software SigmaStat for Windows,version 2.0 (Jandel Scientific, Chicago, IL). Assumptions of normality and homoscedasticity were tested with Kolmogorov-Smirnov and Bartlett tests prior to analysis. Non-parametric methods were used when any of these conditions were violated.

RESULTS

Larval Condition

The initial shell height (median, 322 and 327 μm) and organic content (2.1 and 2.2 μg) of pediveliger larvae were not significantly different in the Spring and Summer batches, yet Spring larvae exhibited significantly heavier and wider shells than Summer larvae (Table 1). Spring larvae also showed higher settlement success (46.5 ± 7.4 spat shell^{-1}, mean and SE) than Summer oysters (29.9 ± 4.5 spat shell^{-1}).

Growth, Condition Index, and Mortality

A decoupling was observed between shell and tissue growth. Shell growth rate was linear throughout the study period (Fig. 2a), whereas tissue accretion was exponential in oysters from the mouth of the bay, with a lag period during the first (Spring) or second (Summer) month of post-larval development (Fig. 2b). Tissue growth was linear in oysters from the head of SQB, but the rate of accretion was significantly lower than the rate of shell growth (Fig. 2, a and b).

Sartas located at the mouth of the lagoon were rapidly covered with sticking sponges, green algae, and bryozoans during the summer months, while those at the head of SQB were essentially free of epibionts. Despite this condition, shell growth rates were higher at the mouth (0.34–0.37 mm d^{-1}) than the head of SQB (0.20–0.23 mm d^{-1}), irrespective of seeding season (Fig. 2a). No significant differences in shell growth rate were observed between spring- and summer-seeded oysters cultured within the same location (Tukey test for multiple comparisons of slopes, mouth q = 1.71, $P > 0.05$; head q = 1.61, $P > 0.05$). Market size (9-cm shell length) was reached after 9 mo post-settlement at the mouth and at an estimated age of 13 mo at the head of the lagoon.

Tissue growth was also higher in oysters from the mouth than the head of SQB (Fig. 2b). At the end of the experiments, TDW was 7- to 8-fold higher for oysters from the mouth (2.10–2.16 g) than the head of SQB (0.23–0.33 g). Juvenile oysters smaller than 15 mm (Spring) or 30 mm (Summer) exhibited a high (>70) CI, but these values dropped to a minimum during the fall and winter months (Fig. 3a). Condition index increased again in oysters from the mouth, but not from the head of SQB, towards the end of the winter (Fig. 3a). Overall, oysters from the mouth consistently exhibited higher CI than those at the head (Fig. 3a). Spring oysters from the mouth also exhibited significantly lower CI than Summer oysters (Mann-Whitney T = 958, $n_1 = 31$, $n_2 = 45$; $P = 0.013$). However, no significant differences were detected in the mean CI between Spring and Summer oysters from the head of SQB (Student t test, $t = 0.400$, $n_1 = 35$, $n_2 = 47$; $P = 0.690$).

Most of the *C. gigas* mortality (54%–68%) took place at the channels within the first month post-settlement. Mortality was negligible (<5%) once the oysters were transferred to the intertidal racks, except in Spring oysters located at the mouth of SQB. This batch exhibited the greatest cumulative mortality during the first 2 mo post-settlement (Fig. 3b). A two-way ANOVA test carried out at the end of the experiment showed significant site ($F = 3.35$; d.f. 1, 31 =; $P = 0.012$), but no significant batch (seeding season)

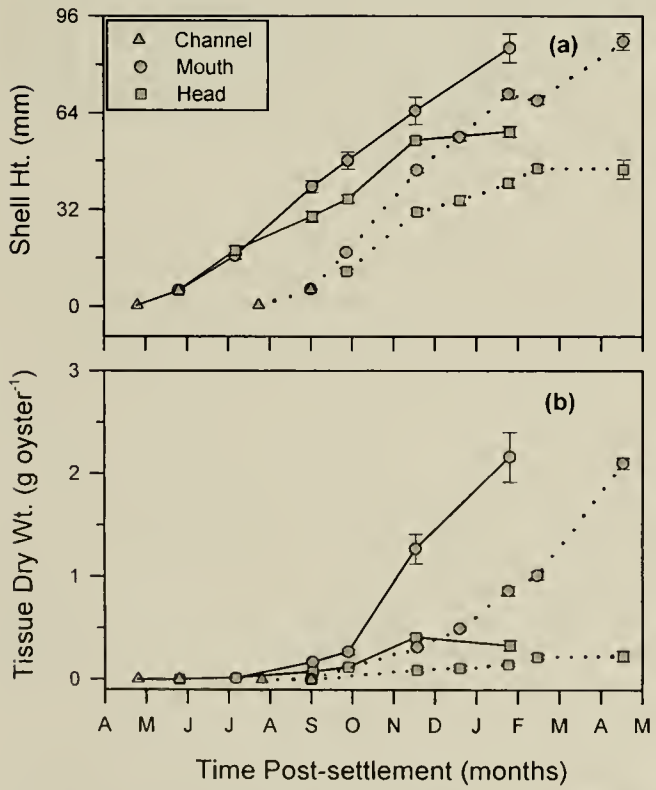

Figure 2. Temporal changes of shell height (a) and tissue dry weight (b) in *C. gigas* cultured at two sites (mouth and head) of SQB. Preliminary rearing (first month post-settlement) was carried out at channels. Data shown for oysters set in April (continuous line) and July (dotted line) of 1995. Vertical bar = SE.

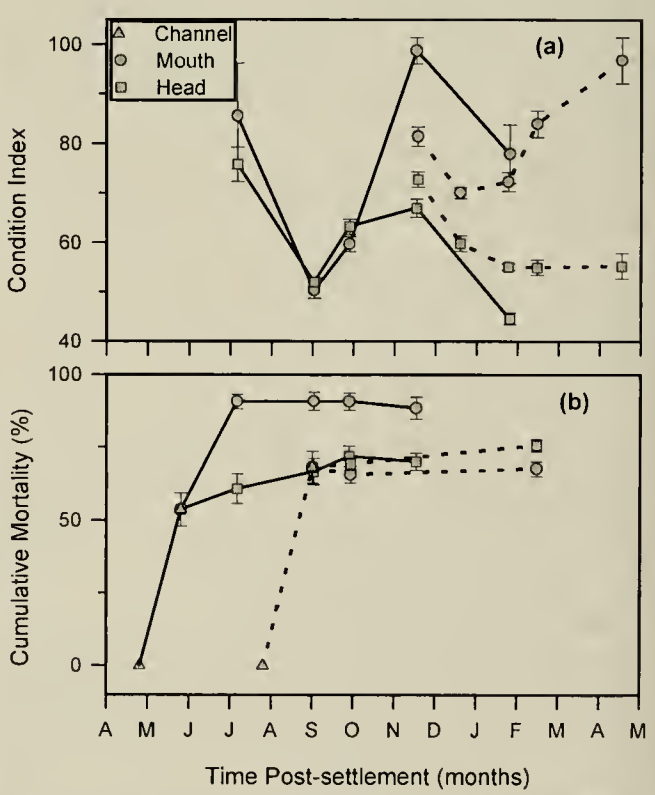

Figure 3. Temporal changes of condition index (a) and percentage of mortality (b) exhibited by *C. gigas* cultured at two sites (mouth and head) of SQB. Preliminary rearing (first month post-settlement) was carried out at the channels. Data shown for oysters set in April (continuous line) and July (dotted line) of 1995. Vertical bar = SE.

effects on the oyster's cumulative mortality ($F = 7.16$, d.f. = 1, 31; $P = 0.077$). A highly significant interaction between both factors was also detected ($F = 21.30$; d.f. = 1, 31; $P < 0.0001$). Overall mortality from setting through harvesting (9-cm shell height) ranged from 68% to 76% (88%, Spring batch from mouth site).

Biochemical Patterns

The amount of proteins, lipids, or glycogen was consistently higher in oysters from the mouth than the head of SQB (Fig. 4). Lipid was the main energy depot during early *C. gigas* development, but glycogen was exponentially accumulated during the fall. Glycogen replaced lipids as the main energy depot when oysters reached a size of approximately 50 mm after 5 mo (mouth) or 7 mo (head) post-settlement (Fig. 4, a–d). When expressed on a percentage basis, lipid and glycogen content were also lower in oyster tissues from the head of SQB, and the lipid/glycogen storage pattern was the same as previously described (Fig.5). Glycogen was accumulated during the early fall, changing from approximately 1% to 21% (mouth) or from 0.3% to 7% (head) during the period from September to November (Fig. 5). During this period, percentage lipid remained relatively constant (4%–5%) in oyster from both sites (Fig. 5). A two-way ANOVA test showed that the percentage of protein did not significantly change with increasing age in oysters from the mouth, yet all three energy substrates (protein, lipid, and glycogen) were significantly affected by tissue type and its interaction with the oyster's age (Table 2). Muscle showed lower glycogen content than tissue remains in oysters from the mouth, but no clear differences were detected in lipids (Fig. 5, a and c). Oyster age, tissue type, and their interaction also affected the relative amount of proteins in organisms located at the head of SQB (Table 2), yet no significant age or interaction effects were detected in lipid content (Table 2). The relative amount of glycogen in oysters from the head was only affected by age (Table 2), with higher values observed at the end of November when glycogen concentration in oyster tissues was highest (Fig. 5, b and d).

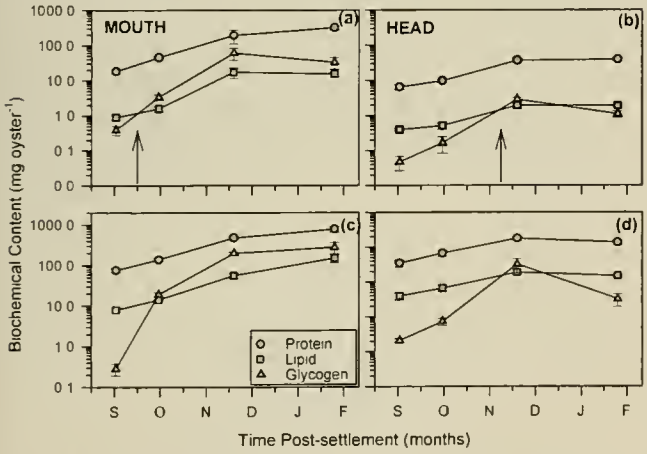

Figure 4. Temporal changes in the absolute amount of protein, lipid, and carbohydrate in the adductor muscle (a and b) and remaining tissues (c and d) of *C. gigas* cultured at two sites (mouth and head) of SQB. Data shown from the fourth month of age through the end of the experiment for oysters set on April of 1995. Arrows indicate the timing when glycogen replaced lipids as the main energy depot. Vertical bar = SE.

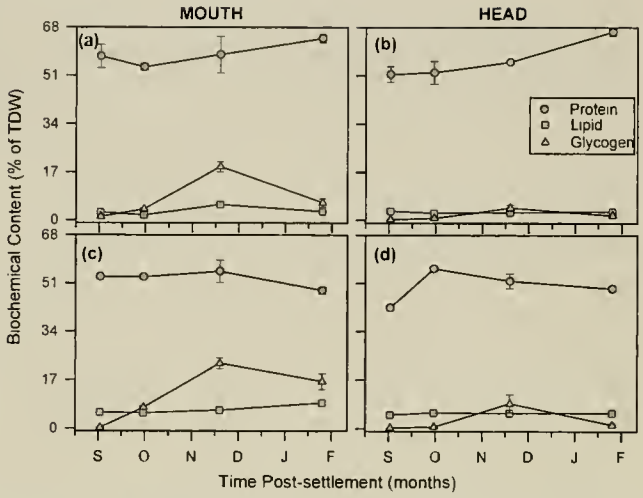

Figure 5. Weight-specific content of proteins, lipids, and carbohydrates in the adductor muscle (a and b) and remaining tissues (c and d) of *C. gigas* cultured at two sites (mouth and head) of SQB. Data shown from the fourth month of age through the end of the experiment for oysters set on April of 1995. Vertical bar = SE.

Environmental Variables

A failure of the thermograph deployed at the mouth of SQB prevented the collection of temperature records at this site after mid-September. However, based on the available data from the previous months, it was observed that the water temperature was consistently colder at the mouth than at the head of SQB (Fig. 6). The temperature followed a tidal rhythm, with stronger effects observed at the mouth of the lagoon. Summer temperature gradually increased at the mouth from 19.3 °C (July) to a peak of 24 °C in September and from 19.5 °C to 24.8 °C at the head of SQB. Daily temperature differences within sites ranged from 1.7 °C to 2.8 °C (mouth) and from 1.7 °C to 2.0 °C (head). It is noteworthy that between-site temperature differences (approximately 1.2 °C) were similar to or lower than the daily variability recorded within sites (Fig. 6).

A trend of increasing TPM was observed at both study sites towards the winter months, with values ranging from approximately 3 mg (June) to 10 mg TPM l^{-1} (February), except in September where an abnormally high TPM value (approximately 32 mg l^{-1}) was recorded (Fig. 7a). No statistical differences in the mean TPM concentration were found between sites (Mann-Whitney, $T = 86.0$, $P = 0.162$). POM remained relatively constant (1.2–2.3 mg l^{-1}) throughout the experiment at both locations, except at the end of August (approximately 0.2 mg l^{-1}) and the end of September, where POM reached up to 6 mg l^{-1} (Fig. 7a).

Estimations of aerial exposure time based on the tidal height at each one of the sites indicated that oysters from the head of SQB consistently experienced longer (23%–26%) immersion time than those at the mouth. In addition, higher aerial exposure was observed in the winter months (Fig. 7b).

DISCUSSION

Growth

One of the most conspicuous observations of the present study was the decoupling between shell and tissue growth during early spat-juvenile development. Shell growth was linear and tissue

TABLE 2.

Two-way ANOVA tests for the effects of oyster age and type of tissue on the relative (percentage) content of proteins, lipids, and glycogen in cultured *Crassostrea gigas* from two sites (mouth and head) of San Quintin Bay. DF = Degrees of freedom; P = probability of rejecting the null hypothesis.

Source of variation	DF	Protein		Lipid		Glycogen	
		F	P	F	P	F	P
			Mouth				
Age	3	0.41	0.751	26.37	<0.001	70.41	<0.001
Tissue	1	7.25	0.016	194.68	<0.001	15.81	0.001
Age × Tissue	3	2.18	0.131	20.12	<0.001	5.03	0.001
			Head				
Age	3	9.56	<0.001	0.47	0.711	12.05	<0.001
Tissue	1	19.57	<0.001	140.68	<0.001	1.09	0.312
Age × Tissue	3	9.39	<0.001	2.14	0.135	1.82	0.184

growth was exponential, with a substantial lag period in oysters from the mouth of SQB. Alternatively, shell and tissue growth were linear in oysters from the head of SQB, but the latter was accrued at a much lower rate than the shell (Fig. 2). Despite the observed decoupling, tissue content was still a major component of the total body weight of oyster post-larvae, as shown by the high condition indices found at these sizes (<4 mm), when compared to juvenile-adult stages (Fig. 3a). The rapid shell growth exhibited by *C. gigas* during early development may have an adaptive significance for predator avoidance (Garcia-Esquivel and Bricelj 1993 and refs. therein), yet the major implication of the observed decoupling may lie on the fact that tissue mass was sensitive to developmental changes and seasonal (temperature) stressors. Thus high values of CI were associated with a relatively high proportion of tissue body mass, typical of early post-larval stages. Garcia-Esquivel (2000) has also shown that shell height of laboratory-reared *C. gigas* spat (0.32–5 mm size range) scaled allometrically with a mass (tissue dry weight) exponent of 2.75, thus confirming that tissue content was a major component of early oyster stages. On the other hand, low CIs coincided with the highest (Spring batch) and lowest (Spring and Summer batches) seasonal temperatures reported for SQB (this study, Alvarez-Borrego and Alvarez-Borrego 1982). It follows that CI values below 60 were indicative of poor physiological condition (high water content) of the oysters in the field, whereas values above 80, typically found during the spring and the fall, reflected a good physiological condition.

Oysters from the mouth reached the market size of 9 cm after 8 or 9 m post-settlement, while those from the head of SQB reached the same size after approximately 13 to 14 mo. These

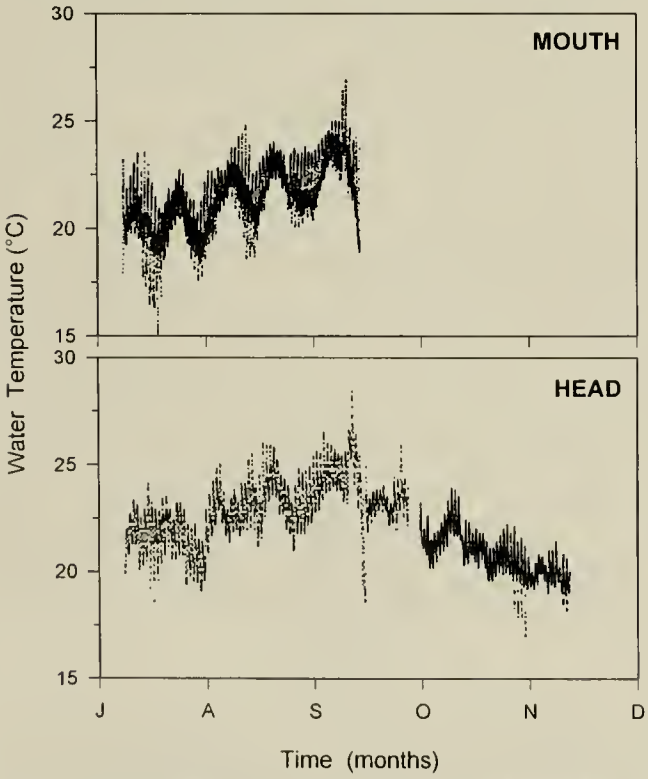

Figure 6. Continuous records of seawater temperature at the head and mouth of SQB during the period of July through November of 1995.

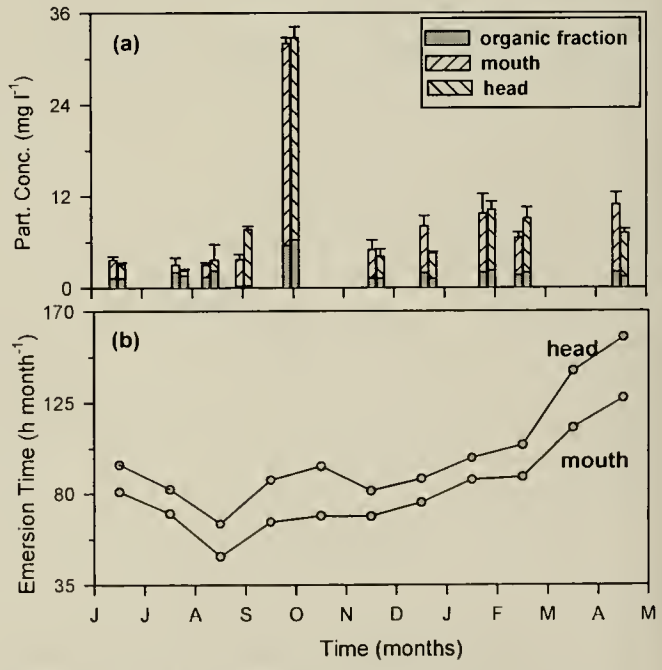

Figure 7. Temporal changes in the amount of particulate matter in the surface seawater (a) and mean aerial exposure time experienced by *C. gigas* at the head and mouth of SQB. The total amount of particles (hatched bars) and organic particles (shadow bars) are shown for each culture site. Vertical bar = SE.

growth rates are higher than those recently reported in the literature. Thus *C. gigas* needed approximately 20 mo to increase their shell height from 1 to 7 cm in tray cultures located at Bahia de la Paz, near the entrance of the Gulf of California (Arizpe 1996), while tray-cultured oysters from the coast of Portugal increased their size from 2 to 8 cm within a period of 14 mo (Almeida et al. 1997). The striking similarities between the growth curve exhibited by the Spring and Summer batches of *C. gigas* within a culture site suggests that seasonal changes of environmental variables were not limiting the growth of this species. However, site-specific differences in growth rates appeared to be associated with microgeographic differences within SQB, since the oyster spat had a common handling history from setting through rearing phases.

Mortality

Spring larvae had heavier shells than the Summer ones. These differences were not reflected in the magnitude of oyster mortalities during the rearing or grow-out phase at SQB, since both batches experienced heavy losses within the first month post-settlement and followed the same pattern thereafter, with clear site effects. The greatest spat mortality was observed within the first month post-settlement at the channels at a size smaller than 6 mm (Figs. 2a and 3b). Although the exact timing of the mass mortality could not be resolved from the approach utilized in this study, detailed laboratory studies have shown that most of the post-larval mortality (up to 50%) of *C. gigas* takes place within the first week post-settlement (Garcia-Esquivel 2000). These mortalities appear to be associated with the amount of pre-metamorphic energy reserves and the degree of their utilization during metamorphosis (Gallager et. al. 1986, Haws et. al. 1993, Garcia-Esquivel 2000), but the lack of biochemical data in these stages prevented any conclusion from the present study.

Mortality during the grow-out phase (intertidal racks) was negligible, and overall losses during the whole production cycle are in agreement with the 50% to 70% mortality previously reported in raft-cultured oysters from SQB (Islas-Olivares 1975, Ramos-Amezquita 1987). The presence of abundant epibionts at the mouth of SQB during the summer suggests that those species could have clogged the filtering apparatus of Spring oysters and were partially responsible for the high post-rearing mortality found at the mouth in this particular batch. Sartas deployed during the summer did not have enough time to get colonized at high densities with these opportunistic species. On the other hand, sartas located at the head of SQB were intermittently exposed to the air, thus preventing any significant fouling throughout the experiments. High mortality of adult oysters (>9 cm) from the outer part (mouth) of SQB has already been reported elsewhere (Terrazas-Gaxiola 1986, Caceres-Martinez et al. 1998). The mortality at this site was partially attributed to the biofouling of encrusting organisms, including the sponge *Halichondria sp.*, the ascidian *Botrylloides sp.*, and the hydroid *Turbularia crocea* (Inclan-Rivadeneyra and Acosta-Ruiz 1989). Despite the potential influence of biofouling organisms and predators on the cultured oysters at SQB, their specific role has not been seriously addressed to date. Recent studies carried out by Caceres-Martinez et al. (1998, 1999) have shown the presence of worms (*Polydora sp.*) near the valve edges of *C. gigas* and around the siphon area of the black clam (*Chione fluctifraga*) from SQB. The authors did not find any statistical relationship between oyster mortalities and the presence of *Polydora sp.*, yet the infestation ranged between 1 and 6 worms per oyster (Caceres-Martinez et al. 1998) and 1 and 48 worms per clam (Caceres-Martinez et al. 1999). Therefore, further studies are required in order to identify the role of potential predators/epibionts on the observed oyster mortality.

Biochemical Patterns

C. gigas followed the same biochemical pattern previously described for the flat European oyster *O. edulis* (Holland and Hannant 1974), with rapid rate of glycogen accumulation after metamorphosis and a shift from lipid to glycogen storage during the fall. The magnitude and pattern of glycogen accumulation appeared to be related with the preparation for *C. gigas* gametogenesis in SQB. Gametogenesis would have been interrupted in the winter when the oysters exhibited a low CI and were forced to utilize endogenous glycogen, resulting in greater energy losses in the oysters at the head (Fig. 4). Although none of the oysters from this study exhibited ripe gonads during the 9-mo experiment, a previous study showed that the reproductive cycle of *C. gigas* at SQB starts with gametogenesis in April, followed by partial and complete spawning throughout July and August (Paniagua-Chavez and Acosta-Ruiz 1995). Accordingly, Mann (1979) found that first maturation of this species was anticipated by a continuous increase of carbohydrates in the tissues, up to a peak coincident with early active and late active stages of gametogenesis (presence of follicles, spermatogonia-oogonia, and spermatocytes-oocytes), whereas a subsequent decrease of glycogen was matched by the presence of ripe organisms. The author did not measure lipids directly, but it can be suggested from the ratio of carbohydrate to total organic carbon that the former was preferentially accumulated only when this ratio was greater than 0.6 (Table 1 in Mann, 1979).

The fall-winter decrease in glycogen content in oyster tissues was apparently due to a combination of low water temperature and lower food availability during this period (see below), whereas the slower growth and lower glycogen content chronically exhibited by oysters from the head of SQB most likely resulted from a combination of frequent reliance on this substrate and shorter feeding periods during aerial exposure, rather than differences in the amount of food available between sites. Throughout the year, oysters located at the head consistently experienced approximately 23% to 26% longer immersion time than oysters from the mouth of SQB (Fig. 7). It is known that *C. gigas* typically shows higher digestive capacity with increasing immersion time, but also reach smaller sizes than those continuously submerged (Moal et al. 1989). Furthermore, there is no evidence of compensatory mechanisms for increasing feeding rates or absorption efficiency with increasing aerial exposure in marine bivalves (Shick et al. 1988). It follows that between-site differences in glycogen content most likely resulted from a combination of a higher utilization of this substrate during anaerobiosis and shorter feeding periods experienced by oysters with longer aerial exposure. In the absence of water, oysters tend to close their valves, are unable to capture food particles, and solely rely on the anaerobic utilization of endogenous glycogen, free amino acids, and proteins to fulfill their energy needs. Bivalves typically depress their metabolism down to approximately 2% to 9% under aerial exposure (Shick et al. 1989) and consequently the cost of anaerobiosis is drastically reduced under these conditions. Therefore, the most significant effect of aerial exposure may not be the utilization of large amounts of endogenous glycogen reserves during anaerobiosis, but the limited

availability of ingested energy to the oysters. This in turn would translate into lower energy available for growth and lower capacity for glycogen storage in oysters subjected to increasingly longer aerial exposure time. Such an explanation is consistent with the findings reported by Moal et al. (1989) and the site-specific differences observed in the present study, in terms of oyster growth and tissue energy content.

Optimal ingestion rates of *C. gigas* are known to occur at 19 °C (Bougrier et al. 1995). On the other hand, continuous temperature recordings carried out at SQB during the experiment showed a temperature maxima of approximately 26 °C in September, and consistently higher values (mean difference of 1.2 °C) at the head. Likewise, Alvarez-Borrego and Alvarez-Borrego (1982) recorded mean annual temperatures of 17 °C at the mouth and 18 °C at the head of SQB, with maximum of 23 °C to 25.3 °C in September and minimum in November and December (12.9 °C to 13.3 °C). Therefore, the temperature regime at SQB seemed appropriate for optimal growth and completion of the storage-reproduction cycle in *C. gigas* throughout the year, except in the winter where the lower temperatures would induce a reduction in the physiological rates (oxygen consumption and clearance rates) of the oysters. It is noteworthy that the absolute amount of TPM steadily increased towards the winter in both study sites, but the POM remained constant (Fig. 7), thus suggesting that higher TPM concentration in the fall/winter resulted, by dilution, in poorer food quality. No clear differences in TPM or POM were found between sites; however, detailed time series previously carried out in the summer over a 10-d period indicated that chlorophyll *a* concentration and productivity was 3-fold higher at the mouth than the interior of SQB (Millan-Nuñez et al. 1982). The discrepancies between both studies may have resulted from the lack of temporal resolution during discrete water samplings and the smaller size (<30 μm) of particles analyzed in this work. In any case, the actual effects of aerial exposure and changes in environmental variables (food and temperature) need to be partitioned in the future, with specific experimental designs.

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SURVIVAL AND FEEDING ACTIVITY OF OYSTER SPAT (*OSTREA EDULIS* L) AS A FUNCTION OF TEMPERATURE AND SALINITY WITH IMPLICATIONS FOR CULTURE POLICIES ON THE SWEDISH WEST COAST

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ABSTRACT This study evaluates the effects of temperature and salinity on feeding activity and survival rate for spat of the European oyster, *Ostrea edulis* originating from the Swedish west coast. The main objective was to investigate local adaptations to hydrographic conditions with low temperatures and salinities with consequences for culture policies. In laboratory experiments, mortality increased at lower temperatures except at salinities below 18 ‰. Highest mortality was found in combinations of very low salinities and the high temperature treatment (10 °C). Feeding activity, measured as fecal production, was significantly lower in 5 compared to 10 °C. Feeding rate began to decline at 28 ‰ and ceased at 16 ‰. The time to recover feeding activity depended upon the duration of low-salinity exposure. Spat exposed to salinities below 16 ‰ did not regain their feeding activity when returned to full salinity, indicating permanent damage. Increased exposure time also lead to higher mortality after the return to full salinity. Comparison between rapid and gradual change of salinity indicates a potential for acclimation. In a model forced by field data on temperature and salinities, survival trajectories were calculated for different water depths and for different seasons. The model predictions were tested in a field experiment where the model could explain winter and spring mortalities in terms of temperature and salinity. In the summer, however, the model underestimates mortality, indicating the importance of such other sources of mortality as fouling and predation. To minimize mortality, this study suggests that culture policies should include deployment below 6 m with initial transfer of spat to the sea at midsummer.

KEY WORDS: *Ostrea edulis*, shellfish, survival, growth, temperature, salinity, aquaculture

INTRODUCTION

The European flat oyster, *Ostrea edulis*, has been used for human food since ancient times, and extensive mariculture is documented from the days of the Roman Empire (Yonge 1960). Excavated piles of oyster shells dating from the bronze age (500–1,500 BCE) bear evidence of a significant fishery on natural stocks of *O. edulis* in Scandinavian waters (Dannevig 1953, Yonge 1960). However, in present times, Scandinavia represents the northern margin of the geographic distribution of *O. edulis*, and climatic changes have probably greatly influenced its local abundance through time. The Swedish west coast, influenced by the Baltic Sea and several river discharges, may be a particularly adverse region with the combination of low salinities and temperatures, which is characteristic of the eastern parts of the seas of Kattegat and Skagerrak. Suboptimal hydrography has been suggested as an explanation for infrequent and irregular recruitment of *O. edulis* along the Swedish west coast (Spärck 1924, Östergren 1925). Nevertheless, the conditions for survival and growth of juvenile and adult *O. edulis* are advantageous with high summer temperatures, high seston concentration (Lännergren 1983, Rödström 1989) and the apparent absence of the lethal parasite *Bonamia ostreae* (Mortensen 1993). Because of the low and irregular natural recruitment, successful exploitation of *O. edulis* along the Swedish west coast will most likely be dependant on land-based spawning and the production of spat for subsequent transfer to the sea. An analysis of the economic prerequisites of a future mariculture development in Sweden will require detailed knowledge about spat survival and growth under local hydrographic conditions. Early life-stages are usually particularly sensitive to suboptimal temperatures and salinities (Kinne 1970, Kinne 1971, Shumway 1996, Walne 1979). The length and variability of these adverse periods will vary with water depth and season. Especially during winter and early spring oysters, will often experience both low temperatures and low salinities.

The objective of the present study was to study experimentally the effect of salinity and temperature on survival and feeding activity of *Ostrea edulis* spat. The experimental results were incorporated into a model driven by local field data on temperature and salinity. The model is used to explore optimal culturing policies with respect to the time of spat transfer and the depth of tray deployment. Finally, the predictions of the model were tested in a field study of spat survival.

MATERIALS AND METHODS

Material

All experiments were carried out using oyster spat produced at the Tjärnö Marine Biological Laboratory (58°, 53′ N, 11°, 8′ E) from controlled spawnings of locally collected adult *Ostrea edulis* L, 1758. Adult conditioning and the culturing of larvae and spat were performed following the protocols described by Walne (1979) and Wilson (1981). After 1 month of conditioning, the broodstock oysters released larvae that were collected on a 90-μm screen. Larvae were then transferred to 34 ‰ filtered seawater (0.2 μm) in 50-L barrels (2 larvae mL^{-1}) and fed the microflagellate *Isochrysis galbana* (Parke) (clone T-iso) at $1*10^5$ cells mL^{-1}. The filtered seawater was maintained at 20 °C, stirred by aeration, and changed every second day. After about 12 days, the larvae developed into competent pediveligers and began to settle on PVC-plates offered as settlement substrate. Larvae that successfully completed metamorphosis were gently removed from the substrate with a razor blade and placed in upwelling columns (diameter: 12 cm, height: 30 cm) with an air-lift drawing water through the

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bottom, which was covered with a 200-μm screen. The up-welling columns with spat were immersed in a 100-L tank with a constant flow of seawater (20 °C, 34 ‰). The spat were grown on a non-limiting supply of *I. galbana* ($1*10^5$ cells mL^{-1}) and were allowed to grow for 6–8 weeks to a shell length of ca 7 mm before they were used in the experiments (except for the field experiment, where spat were 18 mm before transfer to the sea). All cultures of *I. galbana* were grown in batch on f/2 media according to Guillard (1983).

Survival and Fecal production as a Function of Temperature and Salinity

The first series of laboratory experiments were designed to test the combined effects of salinity and temperature on spat survival and feeding activity. The range of salinities tested, 12–34 ‰, covers the range of local field salinities. The experiments were run at 5 and 10 °C, where 5 °C represents a suboptimal temperature at which the oysters are still feeding although at lower rates (Rödström 1989). Befoe the experiments, all oyster spat were kept individually in 100-mL plastic beakers at 34 ‰ and 10 °C for at least 10 days to check that the spat were alive and feeding. All spat were fed before and during the experiments with *Isochrysis galbana* at a concentration of $1*10^5$ cells mL^{-1}. Because field salinities may change rapidly (Fig. 1a), the oyster spat did not get the opportunity to acclimate to the new salinity before the experiment. Field temperature varies more gradually (Fig. 1b), and the spat were acclimated to the experimental temperatures by changing the temperature gradually 0.5 °C per day.

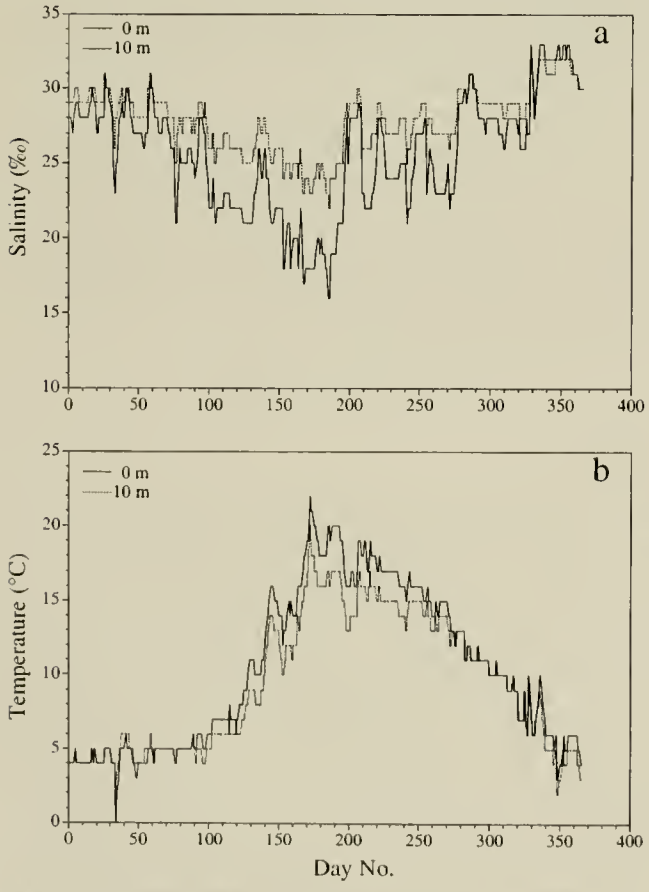

Figure 1. Annual variation in hydrography in surface waters of Tjärnö Marine Biological Laboratory. (a) Salinity (‰). (b) Temperature (°C). Data from 1989 at 0-m and 10-m depth.

The seawater in the experiments was pumped from 40-m depth (34 ‰) and filtered through a sand filter to a 25-m^3 head tank. For salinities lower than 34 ‰, seawater was diluted with deionized and distilled water. The salinity was determined with a temperature-compensated refractometer (accuracy ±1 ‰). Each salinity was mixed 24 h before the experiment and then gently aerated before use. *Isochrysis galbana* was added and mixed in each salinity just before the experiments. During water change, the spat were temporarily drained, while the beaker was rinsed. Then, new seawater with the appropriate salinity and fresh *I. galbana* was added, and the spat were returned to the beaker. Spat were randomly allocated to each treatment, and all beakers were randomly distributed in space. The experiments were carried out in constant-temperature rooms. Every second day, all beakers were examined; an oyster spat was defined dead if it lay open and could no longer close the shell valves when mechanically disturbed.

The effect of temperature and salinity on feeding activity was tested by recording the fecal production, which was assumed to give a time-integrated estimate of feeding activity. Only band-shaped feces were recorded, which were assumed to represent ingested material and not pseudo-feces. The relation between suspension feeding and fecal production was examined in a separate study of clearing rate (see below). The experimental beakers in the survival experiment above were examined every second day, and the amount of feces produced during 48 h was recorded semiquantitatively, where 0, 1, and 2 indicate absence of feces, low feces production, and high feces production, respectively. The experimental treatments lasted for 27 days in 10 °C and for 29 days in 5 °C. After the experiments, all spat were directly transferred back to 34 ‰ and 10 °C, after which the monitoring of survival and fecal production continued for 29 days in 10 °C and 39 days in 5 °C. If no other information is given, 10 replicate spat for each combination of salinity and temperature were used. Daily mortality rates and 95 % confidence intervals were calculated from the slope of the linear regression of the natural logarithm of the number of surviving spat against time. Treatment effects of temperature, salinity, and time on fecal production and interactions among factors were tested with an analysis of variance (ANOVA). The linear model used to describe the scores of fecal production (X) was:

$$X_{ijklm} = \mu + S_i + T_j + W_k + ST_{ij} + SW_{ik} + TW_{jk} + STW_{ijk} + I(ST)_{l(ij)} + WI(ST)_{kl(ij)} + e_{m(ijkl)} \quad (1)$$

where salinity (S), temperature (T), and week (W) are considered as fixed factors, and individual spat (I) is a random factor nested within the factors S and T. The assumption of homoscedasticity was tested using Cochran's C statistic (Winer et al. 1991). In all statistical tests, a type I error rate (α) of 0.05 was used.

Survival and Fecal Production as a Function of Low Salinities

A second experiment was run to investigate in more detail how low salinities affect survival and feeding activity. This experiment was run in the same way as described above, and the salinities tested were 20, 18, 16, 14, 12 ‰ and a control in 34 ‰. However, this time only one temperature (10 °C) was included. After 1 week in 34 ‰ and 10 °C, the oyster spat were transferred to the new salinity directly without acclimation. Spat were exposed to one of the low salinities for 27 days, after which they were transferred back to 34 ‰ and studied for 40 more days. Survival and fecal

production were recorded as described above. Daily mortality rate was estimated with regression analysis.

Survival and Fecal Production as a Function of Low Temperatures

To investigate the effect of very low temperatures on survival and fecal production, two experiments were performed. In the first experiment, survival at the temperatures 4, 2, and 0 °C was studied at 34 ‰ and 22 ‰. The oyster spat were initially acclimated to 10 °C and 34 ‰. Oyster spat were transferred to the different salinities before the experiment started, and the temperature was gradually lowered to each treatment temperature (1 °C day^{-1}). During this period, all spat were kept in the same container (one for each salinity) until the treatment temperatures were reached, when the spat were transferred individually to 100-mL plastic beakers. Ten replicate spat for each factor combination were used. The experiments were performed in temperature-controlled water baths. Water was changed every second day and the spat fed *Isochrysis galbana* ($1*10^5$ cells mL^{-1}). The oyster spat were exposed to the low temperatures for 30 days. Daily mortality rate was estimated with regression analysis, as described above.

The second experiment examined the impact of low temperatures on fecal production in 5, 4 and 3 °C at the salinities 34, 24, and 20 ‰. The oyster spat were individually placed in 100-mL plastic beakers and were initially acclimated to 10 °C and 34 ‰. Before the experiment started, three replicate spat were transferred to the different salinities, and the temperature was gradually lowered to each treatment temperature (1 °C day^{-1}). Fecal production during 48 h was recorded as described above and the score (X) is described with the linear model:

$$X_{ijk} = \mu + S_i + T_j + ST_{jj} + e_{k(ij)} \quad (2)$$

analyzed with an ANOVA with temperature (T) and salinity (S) as fixed factors.

Fecal Production as a Function of Rapid or Gradual Change in Salinities

This experiment was performed to test for an effect of the time-scale of salinity changes on spat feeding activity, measured as fecal production. Gradual acclimation was compared to direct transfer of spat to suboptimal salinities. Also examined was the question of whether previous acclimation to low salinities would increase feeding performance when directly transferred to even lower salinities compared to spat being acclimated at higher salinities.

The oyster spat were gradually acclimated to lower salinities with 1 or 2 ‰ per day. They were fed *Isochrysis galbana* ($1*10^5$ cells mL^{-1}) every second day when the water was changed. All spat were kept in the same container until the target salinity in the specific treatment was reached. After that, spat were individually transferred to 100-mL plastic beakers. Three replicate spat for each factor combination were used. Before the actual recording of fecal production started, the spat were allowed to acclimate to the treatment salinity for 2 more days. For each experimental transfer, three control individuals from a pool of spat kept at 34 ‰ were treated identically and transferred to new beakers with 34 ‰. The temperature during the experiment was 14 °C. Fecal production after 24 h was recorded semiquantitatively on a scale where 0, 1, 2, and 3 indicate absence of feces, low, medium, and high feces production, respectively. The following protocols were used:

1. spat initially acclimated in 34 ‰ and directly transferred to 28, 24, 20, 16, and 12 ‰, respectively;
2. spat initially acclimated in 24 ‰ and directly transferred to 34, 20, 16, and 12 ‰, respectively;
3. spat initially acclimated in 20 ‰ and directly transferred to 34, 16, and 12 ‰, respectively;
4. spat initially acclimated in 16 ‰ and directly transferred to 34 and 12 ‰, respectively; and
5. spat initially acclimated in 12 ‰ and directly transferred to 34 ‰.

Survival and Feeding Activity as a Function of Exposure Time at Low Salinity

Previous experiments suggested that recovery from low salinities was affected by the duration of the exposure. To examine the effect of exposure time on survival and feeding activity, oyster spat were exposed to the suboptimal salinity 16 ‰ for 1, 2, 3, and 4 weeks, respectively. For each exposure period, 10 replicate spat were used. Before the experiment, all oyster spat were kept in 34 ‰ and fed *Isochrysis galbana* ($1*10^5$ cells mL^{-1}) every second day when the water was changed. The spat were then transferred to 16 ‰ directly without any previous acclimation. After the different exposure times, the spat were transferred back to 34 ‰ and followed for another 54, 47, 40, and 33 days, respectively. Survival and fecal production were recorded during the experiment as described above. Daily mortality rates during the recovery phase was estimated by linear regression as described above, and the scores of fecal production (scale: 0, 1, 2) the first week of recovery were tested with a one-factor ANOVA with incubation period as a fixed factor.

Clearing Rate as a Function af Salinity

In the experiments described above, feeding activity was inferred from fecal production. To test the assumed relationship between suspension feeding and fecal production, the actual rate of suspension feeding was studied and compared with measured fecal production. The clearing rate of oyster spat was determined in 34, 28, 24, 20, 16, and 12 ‰ by measuring the disappearance of suspended *Isochrysis galbana* cells exposed to oyster spat. All spat were initially kept in 34 ‰, 14 °C, and fed *I. galbana* ($1*10^5$ cells ml^{-1}). The spat were kept in 100-mL plastic beakers during the experiments. At each salinity, three replicate spat (one spat per beaker) and two control beakers without spat were incubated for 24 h. One sample (1 mL) of the *I. galbana* concentration from each beaker was collected at the start and after 24 h and fixed in Lugol (acid iodine-iodide). The fixed samples were allowed to settle for 24 h in the wells of a multidish (Nunc, 3.5 mL). The wells were then examined with an inverted microscope (Nikon Diavert, 200X) and video-recorded images of *I. galbana* cells were counted with an image analysis software (IPLab, Signal Analytics, Inc.). The volume cleared per unit time (F) of *I. galbana* by the oyster spat was calculated from the decrease in concentration during the incubation according to:

$$F = [\ln(C_t/C_0) - \ln(E_t/E_0)] * V/t \quad (3)$$

where C and E are the concentrations at the start (0) and after some time (t) of the controls and the spat treatments, respectively, and V is the volume of the experimental container. The estimated clearing rates were finally compared to the scores of fecal production.

Model of Spat Survival and Feeding Activity as a Function of Local Hydrography

The significance of the laboratory experiments on spat survival as a function of salinity and temperature during local field conditions was explored in a model simulation. The main objective was to analyze the effects of the time at transfer of spat to the sea and the depth of deployment. Data on temperature and salinity were collected at the Tjärnö Marine Biological Laboratory between 1981 and 1991 (unpublished data). One dataset consists of approximately daily recordings of surface (0.5 m) temperatures and salinities. By combining this time series with a dataset consisting of monthly recordings of vertical (0–30 m) temperature and salinity profiles, a matrix of daily temperatures and salinities was estimated covering every meter extending from the surface to a depth of 15 m. Based on regression analysis, the following relations were used to estimate temperature (τ) and salinity (s), at depth (z) from surface recordings (τ_0, s_0):

$$\tau(z) = (-0.000812 * m^4 + 0.021 * m^3 - 0.173 * m^2 + 0.474 * m - 0.318) * z + \tau_0 \quad (4)$$

$$s(z) = (-0.04426 * s_0 + 1.372) * z + s_0 \quad (5)$$

where m is the month (1 to 12). Validation of these relations showed that error rarely exceeds 10 % for temperature and 5 % for salinity.

From the results of the laboratory experiments on spat survival as a function of temperature and salinity, a survival matrix was constructed (Fig. 2). Daily survival rates for temperatures between −1 to 25 °C and salinities between 11 and 34 ‰ were estimated by inter- and extrapolation from the experimental results. No reduction in survival rates was assumed in salinities between 24–34 ‰ when temperature is above 10 °C (Newkirk et al. 1995). The experiments also revealed that exposure at low salinities (< 18 ‰) imposes a stress load resulting in delayed mortalities when returned to higher salinities. This effect was included in the model as an added mortality when returning to salinities ≥ 19 ‰ according to a loading function estimated from a curve fit to experimental data (see Fig. 4) as:

$$\text{added daily mortality} = 0.03 * [1 - \exp(-0.005 * h^2)] \quad (6)$$

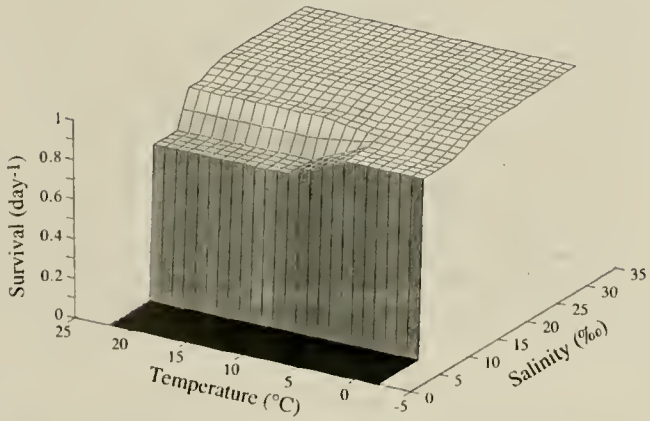

Figure 2. A graphic representation of the matrix of daily survival rate of *Ostrea edulis* spat as a function of temperature (°C) and salinity (‰) used in the model simulations. Survival rates are inter- and extrapolated from experimental results.

where h increases by 1 for each day at salinities < 18 ‰ and decreases by 1 at salinities ≥ 19 ‰ (h ≥ 0). Sensitivity analysis showed that the added mortality had little effect on the simulation results. The forcing from the temperature and salinity matrices generates a time trajectory of daily survival rates that was extracted for each depth, and the total survival over the time period was calculated. Time trajectories for 120 days at three different starting times were explored, April 1, June 1, and September 1. This was repeated for temperature and salinity data for the 9 years between 1981 and 1989.

A less rigorous model was formulated to examine the relative effect of local hydrography on feeding activity. Because feeding activity was recorded as ranked fecal production, the objective was only to compare relative feeding activities for different depths and at different seasons. A matrix of feeding activity as a function of temperature and salinity was constructed from the experimental data. With the forcing from the temperature and salinity matrices (1981–1989), a time trajectory of daily activity was extracted for each depth between 0–15 m, and for the same three starting times as for the mortality simulation. An attempt was also made to combine feeding activity with food availability by multiplying activity scores with local chlorophyll *a* measurements, available in the model as a matrix of monthly vertical profiles calculated from 5 years of field measurements (unpublished data from the Tjärnö Marine Biological Laboratory). The output from the simulations of the feeding activity model is a cumulative sum of daily activity times chlorophyll *a* concentration. Computer simulation of the mortality and feeding activity models was performed in MATLAB® 4.2 (MathWorks Inc) for the Apple Macintosh.

Field Experiment of Oyster Spat Survival and Growth

A field experiment was designed to test how much of the *in situ* survival rate could be explained by the mortality rates observed in the laboratory experiments and predicted by the model described above. The experiment was carried out adjacent to a blue mussel culture outside Grebbestad about 30 km south of the Tjärnö Marine Biological Laboratory. A set of 148 oyster spat (18.3 ± 0.3 mm, mean ± SE, n = 148) were transferred to the sea in November 1988, and survival and growth was followed to November 1991. The spat were cultured in suspended, plastic trays (600 × 400 × 140 mm), stocked at a density of 350 spat m^{-2} and deployed at an average depth of 4.5 m. All spat used were hatched at the laboratory using brood-stock oysters from local populations. The spat were examined in the field for survival and shell length on eight occasions, and at each sampling occasion, the spat were relayed into clean trays. The presence of fouling organisms and potential predators was also recorded.

RESULTS

Survival as a Function of Salinity and Temperature

The mortality rate was highest in the lowest salinities where exposure to 12 ‰ killed all spat within a week (Fig. 3). Salinities lower than 18 ‰ are apparently suboptimal; only 40–50 % of the spat survived 16 ‰ for 30 days. The effect of the temperature reduction from 10 to 5 °C was more complex where there was an increase in mortality below 28 ‰, except for the lowest salinities tested where the effect of temperature was reversed, and mortality increased with temperature (Fig. 3). At very low temperatures (0–4 °C) mortalities were generally low (Table 1). There was almost no

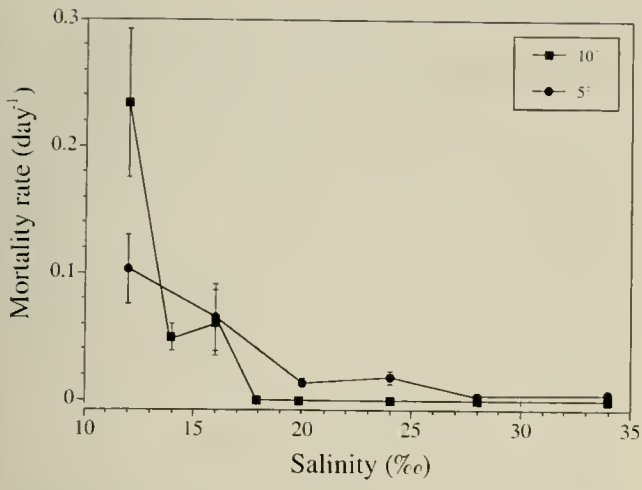

Figure 3. Daily mortality rate (mean ± 95 % CI) of *Ostrea edulis* spat as a function of salinity and temperature.

effect at 34 ‰ and a slight increase of mortality toward lower temperatures at 22 ‰. After exposure to low salinities, the spat were transferred back to 34 ‰ and survival was recorded during this recovery period. During the recovery period, mortality rate was higher for the spat previously exposed to the low salinties 14 and 16 ‰ as compared to spat incubated in higher salinities. An increased mortality during the recovery phase was evident also in the 20 and 24 ‰ treatments for spat incubated at 5 °C. Mortality during recovery declined 1–2 weeks after transfer to 34 ‰. A more detailed study showed that future mortality after exposure to low salinity (16 ‰) is dependent on the exposure time (Fig. 4). Survival was initially high in all four exposure treatments, and a difference in mortality rate was first evident after the spat were transferred to 34 ‰. Mortality dramatically increased after 2 weeks of exposure to 16 ‰.

Feeding Activity as a Function of Salinity and Temperature

Feeding activity in different combinations of temperature and salinity was inferred from studies of the fecal production. Fecal production has the advantage of integrating feeding activity over time and was also easily observed and quantified on an ordination scale. The assumed relationship between what we considered as fecal production and actual feeding rate was tested by measuring the clearing rate of oysters feeding on *Isochrysis galbana*. Figure 5 shows the relation between fecal production (in relative units) and the clearing rate (mL h^{-1} g^{-1}) as a function of salinity at 14 °C.

Feeding activity dropped dramatically after direct transfer of oyster spat from 34 ‰ to salinities lower than 28 ‰ (Fig. 6). Spat in the 18, 20, and 24 ‰ treatments regained their feeding activity

TABLE 1.

Daily mortality rates (mean ± 95% CI) as a function of low temperatures at two salinities.

	Salinity	
Temperature	22 ‰	34 ‰
0°C	0.012	0
2°C	0.0074	0.0030
4°C	0.0035	0

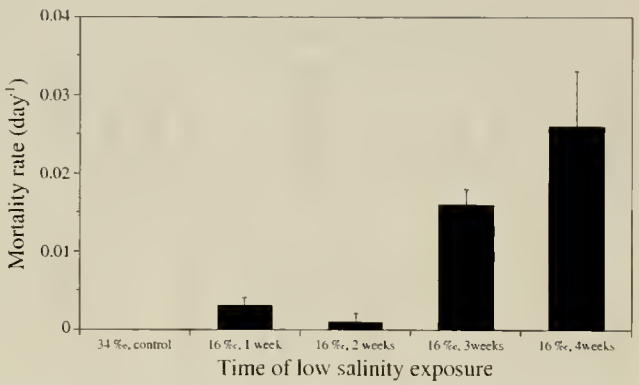

Figure 4. Daily mortality rate (mean ± 95 % CI) of *Ostrea edulis* spat after exposure to low salinity (16 ‰) for periods of 1, 2, 3, and 4 weeks, respectively. Temperature was 10 °C.

after some days; whereas, spat in the 12, 14, and 16 ‰ ceased to feed. The time to regain feeding activity after transfer to 34 ‰ increased at lower salinities, especially in the low temperature treatment. Analysis of variance of the linear model (Eq. 1) shows that there is a significant interaction between the effects of temperature and salinity on feeding activity (Table 2). This is interpreted as a general decrease of feeding activity with lower salinity and that the low temperature treatment (5 °C) further reduces feeding but only at low salinities (Fig. 6). Note that feeding activity at salinities <20 ‰ is not considered in the statistical analysis because of high mortality rates. Feeding activity at low temperatures (3–5 °C) was significantly reduced (Table 3) and completely ceased at 3 °C independent of salinity. The time to recover full feeding activity when transferred back from low to high salinity (34 ‰) increased at lower salinities and could take several weeks (Fig. 6). Similar to the effect on survival, the recovery of feeding activity after exposure to low salinities depended on the exposure time (Fig. 7). After 1 week of exposure to 16 ‰ with no feeding activity the spat quickly recovered when transferred to 34 ‰. As the time of exposure increased so did the recovery phase ($F_{3,31}$ = 6.9, P = 0.001).

Feeding Activity as a Function of Gradual Acclimation to New Salinities

This experiment was performed to investigate how feeding activity responded to a gradual acclimation to low salinities com-

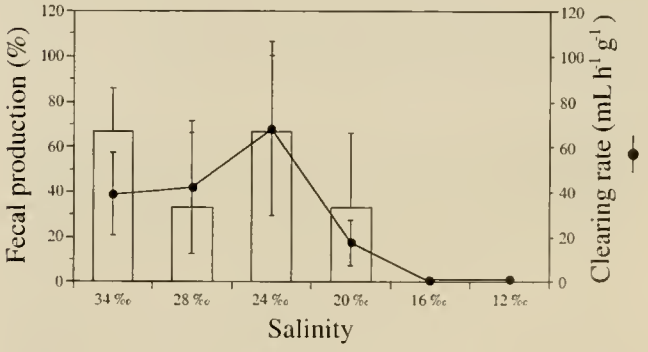

Figure 5. Fecal production (% of maximum score) and clearing rate (mL h^{-1} g^{-1}, mean ± 95 % CI) for *Ostrea edulis* spat in different salinities at 14 °C.

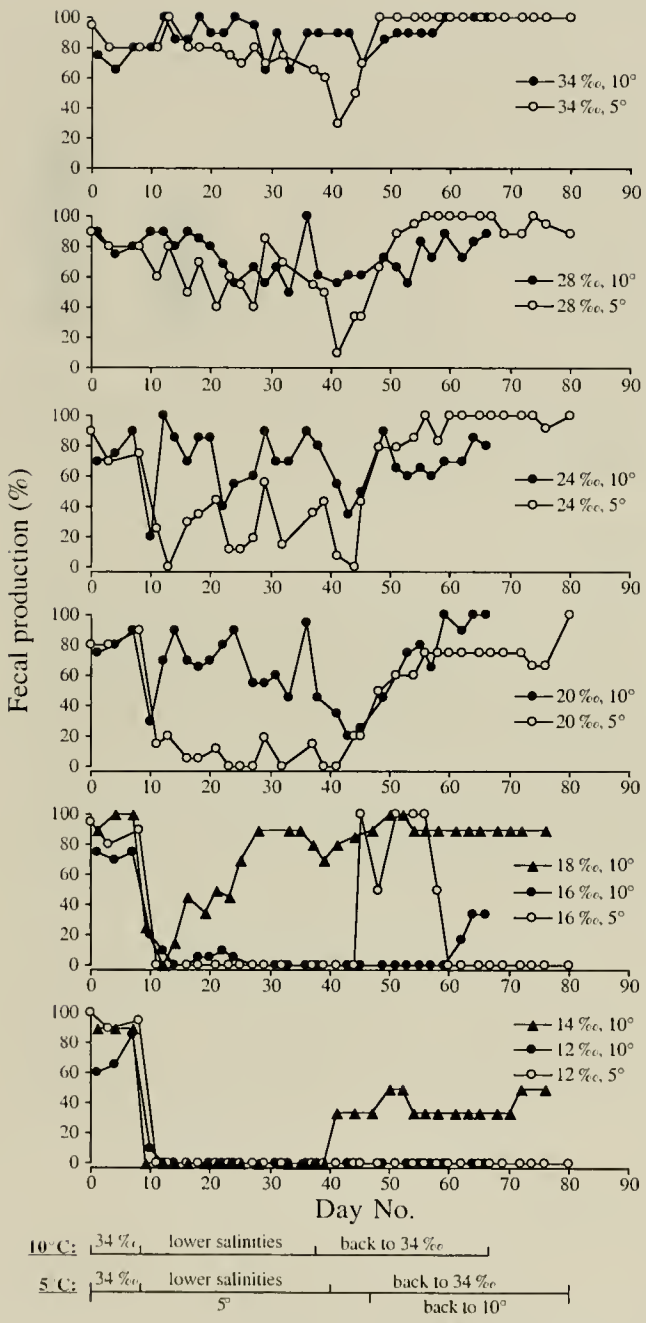

Figure 6. Time trajectories of fecal production (% of maximum score) of *Ostrea edulis* spat in different salinities at 5 °C and 10 °C. Below the panels are transfer protocols for the different treatments.

pared to a rapid change in salinity. Figure 8 shows the feeding activity of oyster spat where each of the five panels represents a salinity that was reached by gradual acclimation. The columns in each panel represent the feeding activity for spat directly transferred to new salinities. The experiment shows that it was not possible to acclimate the spat to 12 ‰. However, spat gradually acclimated to 16 ‰ performed better compared to spat directly transferred from 34, 24, or 20 ‰. Note that the feeding activity of the control spat in 34 ‰ declined over time indicating a general deterioration of spat quality. This should, however, not change the conclusions about the effect of gradual acclimation.

TABLE 2.

Analysis of variance of the effects of temperature and salinity on oyster spat feeding activity, measured as fecal production.

Source	d.f.	Mean Square	F	*P*
Temperature	1	30.0	23.2	<0.0001
Salinity	3	25.9	20.1	<0.0001
Week	3	0.792	1.45	0.23
Individual (Temp., Salinity)	48	1.29	2.81	<0.0001
Temp. * Salinity	3	3.92	3.03	0.038
Temp. * Week	3	0.518	0.948	0.42
Salinity * Week	9	1.49	2.73	0.0057
Temp. * Salinity * Week	9	0.891	1.63	0.11
Individual (Temp., Salinity)* Week	144	0.546	1.19	0.12
Residual	224	0.460		

The factor "Week" identifies variation among different weeks during the 30-day experiment, and the factor "Individual" identifies variation among the studied spat in each combination of temperature and salinity. Temperature levels: 5 and 10 °C; salinity levels: 20, 24, 28, and 34 ‰.

Simulation of Spat Survival as a Function of Local Hydrography

The over-all goal of the present study was to elucidate the effects of low temperatures and salinities in the field on survival and feeding activity of oyster spat. Based on the results from the laboratory experiments, we wanted to extrapolate to local field conditions to obtain information about expected mortalities as a function of the depth of spat deployment and the season of spat transfer to the sea. From hydrographic datasets on temperature and salinity at the Tjärnö Marine Biological Laboratory, survival trajectories were calculated using the mortality rates estimated in the laboratory experiments. Results from simulations of the model for 9 different years of hydrographic data show that both the depth of deployment and the time of spat transfer strongly affect average mortality rate. Mortality rate increases above a depth of ca 8 m, in particular for spat transferred in April (Fig. 9). Mortality is generally highest for spat transferred in April; whereas, it is very low for transfers starting in July. Also note that the variability of mortality among years is highest in the surface waters.

Simulation of Spat Feeding Activity as a Function of Local Hydragraphy

Results from the simulation of the feeding activity model can only be used to infer qualitative effects of depth and timing of spat transfer to the sea. Activity is predicted to increase with depth with more saline water (Fig.10a). Over the 120-day period, cumulative

TABLE 3.

Analysis of variance of the effects of low temperatures and low salinities on oyster spat feeding activity, measured as fecal production.

Source	d.f.	Mean Square	F	*P*
Temperature	2	7.0	14.5	0.0002
Salinity	2	1.0	2.08	0.15
Temp. * salinity	4	0.33	0.692	0.61
Residual	18	0.481		

Temperature levels: 3, 4 and 5 °C; salinity levels: 20, 24, and 34 ‰.

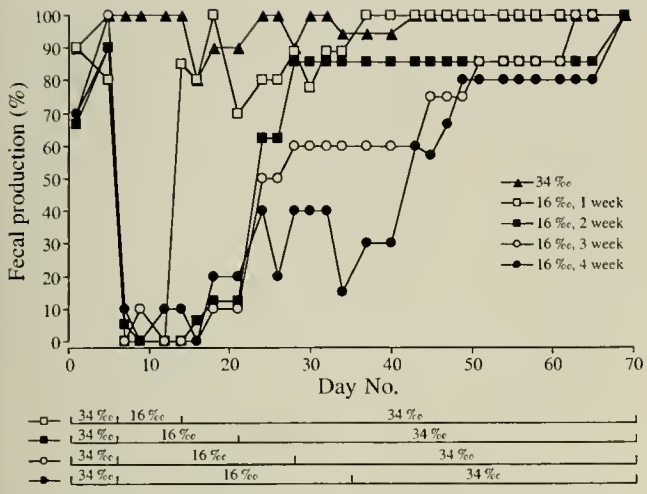

Figure 7. Time trajectories of fecal production (% of maximum score) of *Ostrea edulis* spat during exposures to 16 ‰ salinity for 1, 2, 3, and 4 weeks including subsequent transfer back to 34 ‰. Below the panel are transfer protocols for the different treatments.

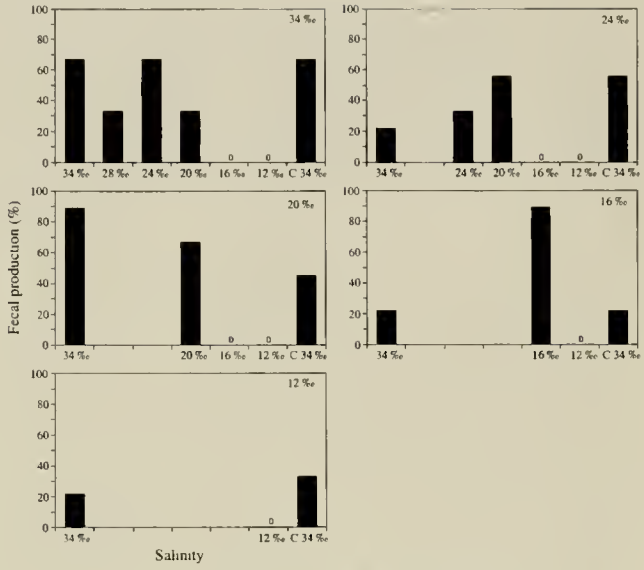

Figure 8. Fecal production (% of maximum score) of *Ostrea edulis* spat after rapid and gradual transfer to lower salinities at 14 °C. Each panel represents the salinity to which larvae were gradually acclimated. Each treatment within a panel represents a salinity to which larvae were rapidly transferred. A control (C 34 ‰) shows the fecal production for larvae which remained at 34 ‰. Treatments with no recorded fecal production are indicated with 0.

activity is greatest for spat starting in July and least for spat starting in April. Because food availability varies both with depth and season, the food uptake will depend both on activity and food concentration. In a coarse attempt to account for food availability, we ran a similar simulation but multiplying activity with a chlorophyll *a* matrix compiled for the depths 0–15 m and for the different seasons (monthly resolution). Inclusion of food availability changes the depth profile, compared to the profile of activity, mainly by reducing the advantage at greater depths (Fig. 10b).

Field Experiment of Oyster Spat Survival and Growth

The daily mortality rate and the shell growth rate of a cohort of oyster spat transferred to the sea in suspended trays are shown in Fig. 11a. Also shown is a simulation of the mortality model based on depth-specific data on temperature and salinity for the time period covered in the field experiment (Fig. 11b). Mortality of oyster spat shows maxima in the summers of 1989 and 1990 with rates not explained by the model. Mortality may have been caused by a combination of the observed fouling by blue mussels, *Mytilus edulis* L., and the presence of predatory sea stars, *Asterias rubens* L.. At other times of the year, field mortalities are similar to what is predicted by the model. As expected, shell growth rate shows a time trajectory with maxima during the summer months and with very low rates during the winter months.

DISCUSSION

Local Hydrography and the Choice of Experimental Conditions

Ostrea edulis disappears when going southwest from the Swedish Skagerrak to the northern parts of the Kattegat (Korringa 1976). Most probably the distribution of *O. edulis* is limited by low winter temperatures and occasional low salinities in this region. Changes in the relative magnitude of river discharge, particularly in the spring, the high-salinity Jutland current, and the brackish Baltic current lead to large variations in salinity often on short time scales. Reproduction of *Ostrea edulis* is negatively influenced by the local variations in hydrography on the Swedish west coast. Low summer temperatures are supposed to prevent gonad maturation and subsequent release of larvae (Spärck 1949, Spärck 1951, Wilson and Simons 1985). According to local oyster fishers, successful spawning and settlement only occur every 6 years. This lack of continuity is the main reason for the recent interest in land-based spawning to improve spat production. Given the potential option to produce spat of *Ostrea edulis* in a land-based hatchery, the present study is focused on the effect of hydrography on spat mortality and feeding activity when transferred to the sea for subsequent culturing.

The response to fluctuating temperature and salinity in marine organisms often depends on the rate of change (Alderdice 1972,

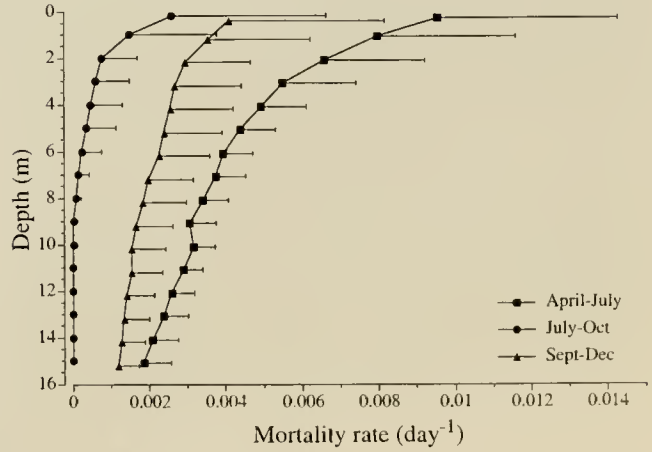

Figure 9. Model simulation of average daily mortality rate (mean ± SD, n = 9) as a function of depth and the time at transfer to field conditions. Three scenarios are simulated, transfer of spat from the hatchery to field conditions in the beginning of April, July, and September, respectively. Time of exposure is 120 days.

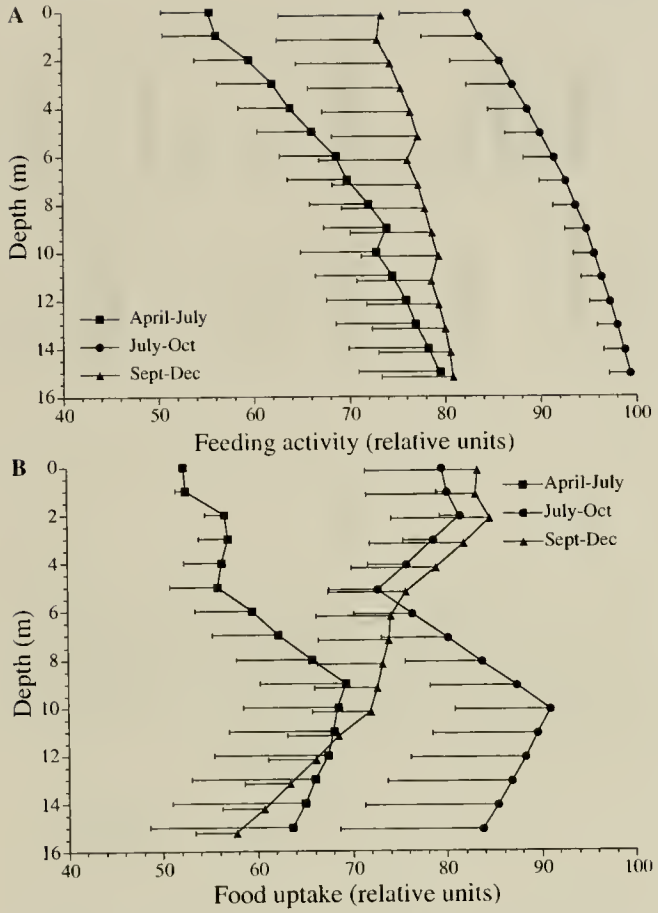

Figure 10. (a) Simulated feeding activity (relative units; mean ± SD, n = 9) as a function of depth (m) and the time at transfer to field conditions. (b) Simulated food uptake (relative units; mean ± SD, n = 9) as a function of depth (m) and the time of transfer to field conditions. Three scenarios are simulated, transfer of spat from the hatchery to field conditions in the beginning of April, July, and September, respectively. Time of exposure is 120 days.

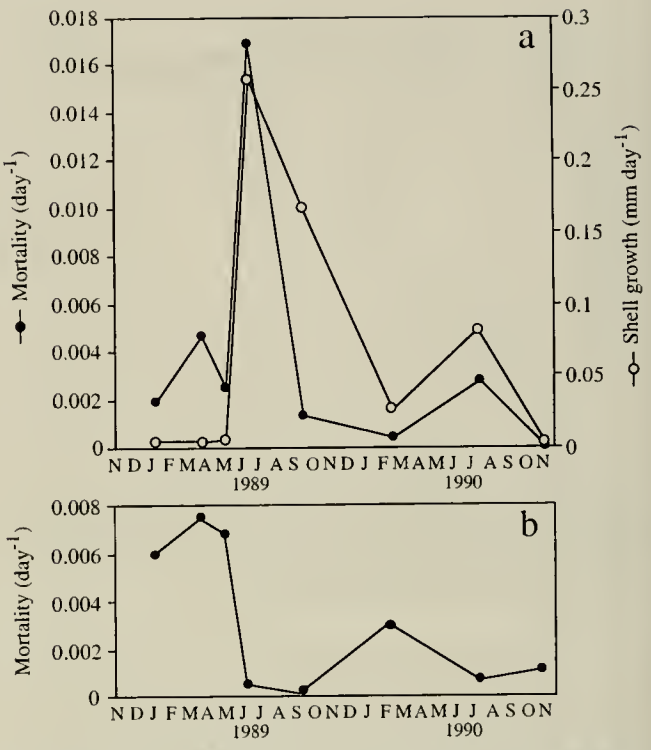

Figure 11. (a) Daily average mortality rate and growth of *Ostrea edulis* spat (mm day^{-1}) in suspended tray culture under field conditions, 1989–1991. (b) Model simulation of daily mortality rate for the time trajectory of salinity and temperature at 5 m during 1989–1991.

Kinne 1970, Shumway 1996, Theede and Lassig 1967). Daily temperature changes along the Skagerrak coast are moderate and rarely exceed 1 °C day^{-1} (Fig. 1b). However, because the Skagerrak coast receives an ever-changing contribution of currents from the North Sea and the Baltic that are further mixed with river input, the salinity may change by several parts per thousand per day (Fig. 1a). The rate of temperature and salinity changes in the experiments of spat mortality and feeding activity were selected to reflect the rate of change observed in the field. Temperature was consequently changed at a maximum of 1 °C day^{-1} while spat were directly transferred between different salinities. The direct transfers between full salinity (34 ‰) and the lowest salinities tested were obviously more rapid than found in the field. The comparison between direct transfer to low salinities and a more gradual change (1–2 ‰ day^{-1}) indicates that oyster spat may maintain feeding activity at lower salinities if the change is gradual (Fig. 8).

Effects of Temperature and Salinity on Mortality and Feeding Activity

Low salinities and temperatures, in the range observed in the field, clearly can increase mortality rate and reduce feeding activity. Daily mortality rates increased sharply below salinities of ca 20 ‰ depending on temperature (Fig. 3, Table 1). Feeding activity declined below 28 ‰ and stopped below 20 ‰. Lowering the temperature from 10 to 5 °C further reduced feeding activity, which ceased completely at 3 °C, independent of salinity (Fig. 6, Table 3). Generally, mortality increased when the temperature was reduced from 10 to 5 °C (Fig. 3). The exception was at very low salinities when mortality increased dramatically in the 10 °C treatment. It may be speculated that the higher metabolic activity at 10 °C (Child and Laing 1998, Shumway 1996) forced the oyster spat to feed more often, resulting in more frequent exposure to the lethal salinity. In contrast, at low temperatures the spat could remain closed for longer periods of time escaping the deleterious effect of ambient low salinity. This is supported by the findings in Hutchinson and Hawkins (1992) that the scope for growth declined in combinations of high temperatures and low salinities. Based on experimental comparisons (Fig. 8) between gradual and rapid changes in salinity, average feeding rate will not depend strongly on the rate of change (max. 3–4 ‰) of field salinities (Fig. 1a).

Long-Term Effects of Low Salinity Exposure

The duration of the exposure to the suboptimal salinity 16 ‰ affected subsequent mortality and feeding activity of spat when transferred to full salinity (Figs. 4 and 7). When exposed for up to 2 weeks, spat rapidly resumed their level of feeding activity, and no increased mortality could be detected. However, when exposure exceeded 2 weeks, there was a lag period for up to 2 weeks before the normal level of feeding activity was attained. A similar effect of long exposure to 16 ‰ was found for spat mortality that continued to be high after transfer to full salinity. This pattern may be explained assuming that spat can close their valves for a limited time when encountering such adverse conditions as low salinities. After some time, depending on physiological conditions, the spat

are forced to ventilate and feed with risks of damage and mortality (Nell and Paterson 1970).

Earlier observations from the Skagerrak coast by Wollebæck (1903) and Dannevig (1953) showed extensive mortalities during March to June when oysters were exposed to low salinities and low temperatures. Similar observations have been reported recently by local fishers on the Swedish west coast. Newkirk et al. (1995) also observed that some areas in Nova Scotia, Canada, were unsuitable for *Ostrea edulis* because of suboptimal periods of low salinity and low temperature. Mortality during cold winter temperatures is, however, apparently low. It may be speculated that the combination of increasing temperature and low salinity will cause high spring mortality because of an increase in metabolic demand forcing the oysters to feed frequently and expose tissues to deleterious salinities. It is also possible that increased spring mortality is caused by a previous cold stress period (Newkirk et al. 1995).

Fecal Production and Clearing Rate

Feeding activity was measured as fecal production, which was quantified on an ordinal scale. The rationale is that fecal production integrates feeding over time, is a direct measure of feeding activity, and it is easy to measure. The correlation between estimated clearing rates and observed fecal production was generally good (Fig. 5). Estimated clearing rates for 12-mm spat feeding on *Isochrysis galbana* were 8–20 mL h^{-1} at 14 °C. This compares reasonably with; for example, Walne (1972), who reported clearing rates for 10-mm spat of 25 mL h^{-1} at 21 °C.

How Much of Field Observations Are Explained by the Mortality Model?

A critical question in the present study is whether the experimental results of mortality and feeding activity can be extrapolated to field conditions and, if so, whether conclusions could be drawn about the expected significance of observed mortality and feeding patterns. As an attempt to validate the experimentally estimated mortality rates, we exposed spat to field conditions for 2 years and monitored mortality and growth (Fig. 11a). Using a mortality model based on the mortality experiments and field data of temperature and salinity, we simulated mortality patterns expected for the field experimental period (Fig 11b). The field experiment showed high mortalities in April and July for spat during their first year. The model correctly predicts the mortality rates found in the spring but strongly underestimates rates in the summer. The high mortality in the field experiment during summer may have been caused by a combination of intense biofouling or predation. In conclusion, temperature and salinity may explain mortality rates in winter and spring, but biological interactions become more important during summer and autumn. It can also be seen that spat suffer less mortality during their second year. This may be explained by the usually increasing tolerance against suboptimal temperatures and salinities with age (Walne 1979), possibly mediated by a greater capacity to remain closed and tolerate starvation. A further explanation could be size-dependent predation.

The growth rates found in the field closely follow predictions from the laboratory experiments on feeding activity as a function of temperature and salinity. Most of the growth occurs between May to November (Fig. 11a). April and May, when food is abundant, give poor growth possibly caused by the combination of low salinities and relatively low temperatures. This view is also supported by the model simulation of feeding activity during this period (Fig. 10a).

Recommendations for Management of Cultures

To explore the significance of observed mortality and feeding patterns for the management of spat transfer to the sea and subsequent culture, we simulated different management policies. By using the model of mortality and feeding activity based on laboratory experiments and field data on hydrography, we simulated transfer of spat at three different seasons and at 16 different water depths (Figs. 9, 10a). The simulations show that mortality is high during spring above a depth of 6 m. The variability among years also increases sharply in the surface waters. We recommend that spat be kept below 6 m during winter and spring to reduce mortality. Of course, a flexible policy would be to take advantage of the year-to-year variability in hydrography by continually adjusting culture depth to prevailing conditions. However, considering the rapid changes in hydrography, this approach would probably be prohibitively expensive. Around July and through summer, the spat mortality caused by temperature and salinty is at its minimum throughout the water-column. Feeding activity may also be at its maximum during this period. Because food uptake is expected, from field data on chlorophyll *a*, to be high regardless of depth (Fig. 10b), an option exists to escape the heavy fouling at the surface during this period (unpublished data) by deploying the spat below 6 m. In the autumn, mortality increases slightly but is still almost independent of depth. Because food uptake is expected to decline with depth (Fig. 10b), we recommend that spat be kept above 6 m during this period. Finally, because local hydrography may differ on small spatial scales because of the extensive archipelago and local land run-off, the selection of culture locality may also be important.

Populations of *Ostrea edulis* on the Swedish west coast experience extreme hydrographic conditions. This may have selected for local tolerance to low temperatures and salinities. Future work should test for the existence of local genetic populations and compare geographical differences in tolerance. This knowledge will be particularly important for management policies of introductions of *O. edulis* from areas with less extreme hydrography.

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AN *IN SITU* STUDY ON THE SURVIVAL AND GROWTH OF *CRASSOSTREA VIRGINICA* JUVENILES IN BON SECOUR BAY, ALABAMA

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ABSTRACT Experimental plots were established at a relic oyster reef on the eastern side of Mobile Bay, Alabama between July 1998 and November 1999 to determine whether elevated beds might improve oyster survival and growth. Oysters (*Crassostrea virginica*) were spawned in a hatchery and the spat were allowed to settle on small oyster shell fragments and on whole oyster shell. Two-month-old juveniles (15–18 mm) were deployed in polyethylene oyster bags on bottom and on underwater shell pads 20 cm and 40 cm above bottom. Oysters on whole shells were deployed outside bags in order to evaluate predation. Remote sensing data loggers were deployed near bottom and 40 cm above bottom to measure temperature, salinity, and oxygen concentration. Growth (increase in height), survival, and condition of oysters in bags at the three experimental depths were compared. Temperature and salinity varied between 11.8 °C–32.8 °C and 4.4 ppt–29.7 ppt, respectively. Periodic anoxic events, which lasted from a few hours to 6 days, were documented. Oysters at the three experimental levels grew to approximately 55 mm during the first year. Total mortality was observed at all three levels during the second summer when oxygen levels dropped to 0 mg L^{-1} for five consecutive days while water temperature was 28 °C. If the 17 months monitored during this study are typical, restoration of Fish River Reef will be difficult due to periodic anoxic events.

KEY WORDS: oyster, oxygen, hypoxia, anoxia

INTRODUCTION

Bon Secour Bay is an embayment located in the southeastern segment of Mobile Bay, Alabama (Fig. 1). Although, some areas of Mobile Bay support a viable oyster fishery, Bon Secour Bay has no commercially harvestable oyster reefs today. Ritter (1895) suggested that overfishing depleted this part of the bay of harvestable oysters. Various reasons have been proposed to explain why viable self-sustaining oyster populations have not returned to the region. Explanations have ranged from the subsidence of the Gulf Coast tidal region to deforestation of the surrounding drainage basin causing an increase in freshwater and silt input (Mackin 1951). Others have suggested an increase in the number of oyster drills, *Stramonita haemastoma* (Eckmayer 1979). Eckmayer (1983) reported a near total mortality of oyster spat in Bon Secour Bay in 1979 due to a freshet. Cake and Eckmayer (1982) summarized reasons for poor production as reduced spatfall, periodic oxygen depletions and hurricanes. Oxygen depletions in August 1967 and July 1978 have also been blamed (Eckmayer 1979).

A peculiar phenomenon that occurs sporadically on the eastern side of Mobile Bay is the "jubilee." During jubilees, considerable numbers of demersal fish and crustaceans are driven toward the eastern shores of the Bay for several minutes up to a few hours (May 1973). Jubilees are wind driven events that result in low oxygen water masses moving eastward along the bottom, forcing benthic animals in front of them. They typically occur in the summer under specific water and wind conditions (Loesch 1960, May 1973). Information necessary to predict where and when jubilees might happen, how long they last, and the height of the anoxic layer above the bay bottom is lacking. Moreover, the periodicity of anoxic conditions in Bon Secour Bay, and any impacts on oyster survival is not known.

The goal of the present study was to determine the feasibility of restoring a relic oyster reef at the Fish River site by the State of Alabama. We measured water parameters (oxygen concentration, temperature, salinity) continuously for several months. We tested for correlations between *in situ* water parameters and juvenile oyster survival and growth. Hatchery produced juvenile oysters were deployed at the experimental site at various depths and the null hypothesis that oyster survival and growth on 20 cm and 40 cm raised platforms was equivalent to survival and growth of oysters deployed on the bottom was tested.

MATERIALS AND METHODS

Research Site and Protocols

Eastern oysters, *Crassostrea virginica*, were strip-spawned and the larvae reared at the Louisiana State University oyster hatchery at Grand Isle, Louisiana. Larvae were divided into two batches; one was allowed to settle on crushed oyster shell and one on whole oyster shell. Juvenile *Crassostrea virginica* were reared for approximately 2 mo in a flow-through system. Ten underwater platforms were constructed at the Fish River Reef (FRR) (Fig. 1) in Bon Secour Bay, using 4-mm thick plastic sheets, either 20 cm or 40 cm wide, and 360 cm long. The plastic sheets were rolled lengthwise and the ends connected to form cylinders with a diameter of 113 cm and a crossectional area of 1 m^2. Plastic mesh was tied across the bottom of each cylinder. The cylinders were then placed next to each other on bottom at the study site (2.8 m deep) and filled with oyster shell so that platform heights were 20 cm and 40 cm above bottom. Shell material was also dispersed on the bottom around each platform. Oysters were deployed on bottom and on the platforms at the two heights on July 30, 1998 and on August 15, 1999.

Remote continuous-monitoring devices (RCMD; Minisonde and Datasonde 3 by Hydrolab) were deployed close to the platforms from July 30, 1998 through February 2000, at 3 cm and 40 cm above bottom in order to monitor oxygen concentration, temperature, and salinity in the vicinity of the deployed oysters. No

Figure 1. Mobile Bay. Point "A" is the approximate location of Fish River Reef.

RCMDs were deployed from November 1998 till February 1999, and only one was deployed at 20 cm above bottom during February and March 1999. RCMDs were exchanged and calibrated twice weekly. A water column profile of temperature, salinity and dissolved oxygen was taken using a YSI 55 hand-held instrument whenever the RCMDs were exchanged. Wind speed and direction data were obtained from the Alabama Weather Information Service.

First Deployment

Two-month-old oysters produced in the hatchery and retained on a 12.5-mm mesh were deployed on July 30, 1998. Six polyethylene oyster bags (12.5-mm mesh) were filled with approximately 500 oyster juveniles (15.2 mm ± 0.44 SE) and deployed on the platforms. Two bags were deployed on bottom, two were deployed on the 20 cm high platforms and two on the 40-cm high platforms. The oysters were then sampled monthly between August and November and bimonthly thereafter. During sampling, approximately 30 oysters from each treatment (height above bottom) were placed in a marked container and returned to the lab. The oyster bags were brushed and shaken vigorously under water in order to remove deposited silt. The presence or absence of fouling organisms and predators was documented. Growth was estimated as the difference between the average height (hinge to opposite margin) of oysters in a sample and the average height of oysters in the preceding sample. On June 8, 1999, the oyster bags were brought to the surface, counted, and live oysters from the two bags at each height were combined into a 25-mm mesh bag and redeployed to the same platforms. Percent survival was estimated from the ratio of live to dead oysters. Hemolymph was taken from ten oysters and checked for Dermo, *Perkinsus marinus*, using the fluid thioglycollate method described by Ray (1952). Intensity of *P. marinus* infection was assessed according to Mackin (1962).

Exposed shell strings were used to control for mortality from predation. Oysters that were allowed to settle on whole oyster shell were counted and a hole was drilled in each oyster-shell substratum. Five shells were attached together by passing a string through the holes and tying a knot on either side. Ten shell strings were then deployed at each depth and held in place by inserting brass clips into holes drilled in the sides of the platforms. A shell string from each treatment was removed and transported to the lab at the same time samples were taken from the oyster bags. On June 30, 1999, the oyster shell attached to strings were found to have wild *C. virginica* juveniles settled on them and were replaced with new shell strings carrying known numbers of hatchery spawned juveniles on July 6.

On June 8, 1999, the height of oysters from the bottom treatment and from the 40-cm platform was measured, and the oysters were cleaned, weighed, and their condition index determined according to methods suggested by Crosby and Gale (1990).

Second Deployment

A second batch of 2-month-old oysters were sieved through a 12.5-mm mesh and the retained oysters were deployed on the platforms on August 6, 1999. Three 12.5-mm mesh oyster bags were filled with 300 juveniles (17.99 mm ± 0.36 SE) each. One bag was deployed at each of the three experimental levels at the study site. During sampling, bags were lifted onto a boat and live and dead oysters were separated and counted. A random sample of 25 live oysters was taken back to the lab for additional measurements. In the lab, oysters were cleaned of all epibionts, blotted dry, weighed, and their height measured.

Average heights and weights of oysters at each depth were compared using one-way ANOVA. We tested the null hypotheses that mean oyster height and weight on the 20-cm and 40-cm platforms was equivalent to mean height and weight of oysters deployed on bottom. Oxygen concentration, temperature and salinity on bottom and 40 cm above bottom were plotted in an attempt to show a relationship between various environmental parameters and survival and growth.

RESULTS

First Deployment

Oysters grew from 15.2 mm to more than 50 mm during the first year. On August 12, 1998 there was no significant difference in height between treatments (Table 1). At the subsequent two sampling dates, oysters on the 40-cm high platform were significantly longer than oysters on bottom ($P < 0.05$). The sample collected on November 24 suggested equal growth of oyster juveniles on bottom and 40 cm above bottom. However, oysters on bottom were thin and elongated (coons). Oysters collected 40 cm above bottom were more circular and deeply cupped. These observed

TABLE 1.

Height (mm) of oysters deployed on bottom and 20 cm and 40 cm above bottom (mean ± SE) at Fish River Reef on July 30, 1998

Date	Bottom	20 cm	40 cm
30 July 1998	15.20 ± 0.44	15.20 ± 0.44	15.20 ± 0.44
12 Aug. 1998	15.10 ± 0.39	16.50 ± 0.55	15.90 ± 0.25
08 Sep. 1998	19.70 ± 0.47	22.20 ± 0.71	22.20 ± 1.05
06 Oct. 1998	26.40 ± 0.88	26.90 ± 0.85	31.50 ± 1.29
19 Jan. 1999	52.24 ± 1.97	42.92 ± 1.58	52.06 ± 1.66
03 May 1999	52.28 ± 2.09	52.97 ± 1.89	56.06 ± 1.43
08 June 1999	56.40 ± 1.45	56.57 ± 1.86	54.20 ± 1.64
13 Aug. 1999	56.22 ± 1.80	51.37 ± 2.04	54.24 ± 2.02

morphological differences remained throughout the study. On January 19, 1999, oysters on the 20-cm platforms were significantly shorter than oysters at the other two levels, but this difference disappeared in subsequent samplings. Height, length, width, and condition index of the oysters collected on June 8, 1999, approximately 1 yr after planting, are presented in Table 2. The data suggest that meat weight and condition of oysters at 40 cm above bottom were significantly greater than weight and condition of oysters on bottom. Oyster survival on June 8, 1999, was similar on bottom (84.5%) and 40 cm above bottom (74.9%) ($P < 0.05$). Dermo intensity varied between 2 and 3 on the Mackin (1962) scale. During routine visual inspections on July 6, most oysters appeared to be alive. On August 13, 1999 all the oysters at the three treatment levels were found dead.

Algal and bryozoan fouling was rare throughout the study. No predators were observed from August 1998 until June 1999. On June 8, 1999, egg cases of the oyster drill, *Stramonita haemastoma*, were found on the bags and juvenile drills were found within the bags at all subsequent inspections. Occasional blue crabs were also encountered after June 8, 1999. No signs of predation were observed on the shells deployed outside the bags.

There were no differences in oxygen, temperature and salinity measurements on bottom and 40 cm above bottom during the study. Temperature ranged from a low of 12 °C in February to a high of 32 °C in August. Salinities generally ranged from lows of 5 ppt to highs of 15–20 ppt. Spikes above and below these ranges occurred but were infrequent. Oxygen concentrations at both depths were also similar, generally ranging from lows of 0.5 mg L^{-1} to highs of 5 mg L^{-1} and greater. There were several instances during which oxygen concentrations on bottom were lower than 1 mg L^{-1} while oxygen concentrations at 40 cm above bottom were greater than 1 mg L^{-1}. These instances were usually of short duration, rarely lasting more than 24 h.

On three occasions, oxygen concentrations at the two depths were near 0 mg L^{-1} for extended periods of time. Between February 25 and February 27, 1999 (Fig. 2) oxygen levels were near zero. Wind speed during that period varied between 1.6 km h^{-1} and 19 km h^{-1} and wind direction varied on an hourly basis. Water temperature during this period ranged between 13.5 °C and 15 °C. On July 16, 1999 (Fig. 2) oxygen levels dropped to near 0 mg L^{-1} and did not rise above 0.5 mg L^{-1} until July 22. Wind speed ranged from 0 km h^{-1} to 15 km h^{-1} changing provenance constantly, and water temperature was about 28 °C. On August 4, oxygen levels dropped to zero where they remained until August 9 (Fig. 2). Wind speed and direction were variable and temperature was above 30 °C.

Throughout the study, including the three extended low D.O. events, oxygen level dropped when salinity increased (Fig. 2). Even a 5-h rise in salinity coincided with a dip in oxygen concentration. As soon as salinity levels fell, oxygen concentrations rose again. The lowest salinity encountered during the study was 4.4 ppt on February 22, 1999, coinciding with a bottom oxygen concentration of 9.31 mg L^{-1}. The highest salinity encountered was 29.8 ppt on September 21, 1999, coinciding with bottom oxygen concentrations of 0.18 mg L^{-1}. Water profiles depicted in Figure 3 indicate a strong pycnocline on July 21, 1999, during a period of anoxia near the bottom. Stratification was observed during hypoxic events and absent when bottom waters were aerobic.

Second Deployment

The oysters deployed on August 6, 1999 had grown by approximately 19 mm during the first 83 days ending on November 1, 1999 (Table 3). There was no significant difference in height between treatments ($P < 0.01$) at any sampling event. On November 1, 1999 survival was 38% on the bottom, 66% at 20 cm, and 69% at 40 cm above bottom. On February 22, 2000 there was no significant difference in oyster height among treatments ($P < 0.02$). Oyster heights were 44.9 mm, 50.4mm and 49.6 mm on bottom, 20 cm and 40 cm, respectively. Survival was 29% on bottom, 48% at 20 cm, and 52% at 40 cm. Seventy-three percent of the dead oysters had a predator hole on one of their shells.

Inspections of the bags during samplings revealed that the bags

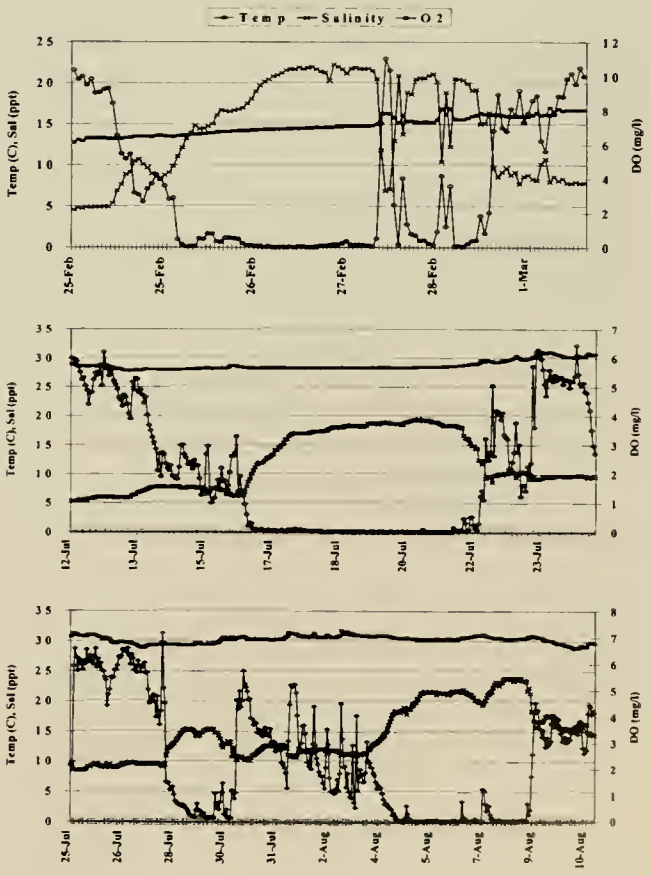

Figure 2. Bottom temperature (°C), salinity (ppt) and oxygen concentration (mg L^{-1}) at Fish River Reef during low oxygen events in 1999.

TABLE 2.

Height (mm), LW (g), SW (g), DW (g), and CI of oysters grown for 1 yr on bottom and 40 cm above bottom at Fish River Reef in Bon Secour Bay, Alabama*

	Height	LW	SW	DW	CI
Bottom	57.00 ± 3.95	21.82 ± 3.08	15.46 ± 2.06	0.46 ± 0.09	72.45 ± 4.72
40 cm	54.20 ± 2.25	23.12 ± 2.88	16.44 ± 2.08	0.57 ± 0.06	87.36 ± 3.53

Measurements are means ± standard error.
* Abbreviations: LW, live weight; SW, shell weight; DW, dry tissue weight; CI, condition index.

on bottom had become covered with a layer of mud and silt while the bags on the platforms remained clean. No morphological differences were observed between oysters at the various depths. Oxygen concentration was near zero the first two days that the oysters were deployed but did not go lower than 0.5 mg L^{-1} for more than a few hours between August 6, 1999 and February 22, 2000.

DISCUSSION

This study documents periodic long-term hypoxic events on the eastern side of Mobile Bay. Oxygen concentrations were measured every half-hour, which allowed documentation of all diurnal fluctuations in oxygen concentrations. Water parameters on bottom and 40 cm above bottom were equivalent throughout the study. Austin (1954) and Ryan (1969) reported flood tides moving eastward and northward along the eastern side of the Bay and ebb tides moving southward along the western side. According to May (1973) and Turner et al. (1987), density stratification causes the isolation of bottom waters in Mobile Bay, and high biochemical oxygen demand (BOD) in the sediment leads to oxygen depletions. Based on these reports, we believe that during periods of stratification, salt water entering Mobile Bay with high tide remains below the pycnocline and gets depleted of oxygen as it moves towards Fish River Reef. These anoxic conditions are different from jubilees that occur north of FRR and are a result of atmospheric conditions that cause the upwelling of anoxic waters that form in low-lying areas of the bay bottom.

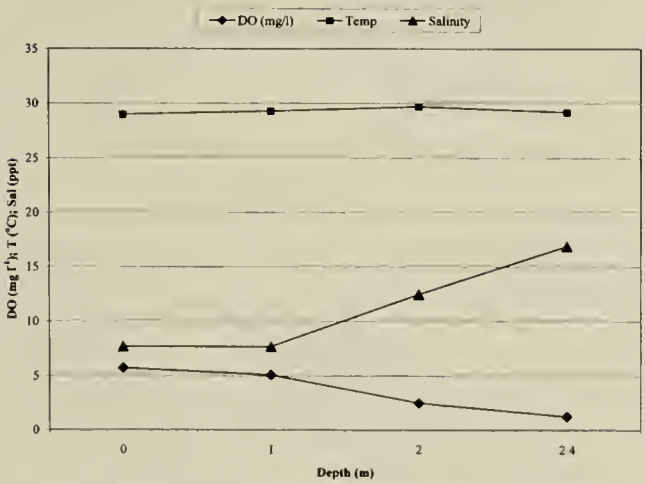

Figure 3. Water column profile of dissolved oxygen (mg L^{-1}), salinity (ppt) and temperature (°C) at Fish River Reef on 21 July 1999.

Two-day hypoxic and anoxic conditions coincident with low temperatures (February 1999, Fig. 2) did not cause oyster mortalities. Five-day anoxic conditions in conjunction with high temperatures (28.5 °C) such as occurred in July 1999 (Fig. 2) caused oyster die-offs. Oxygen depletions in 1971 were blamed for oyster die-offs in Mobile Bay (May 1972). Eckmayer (1979) suggests that oxygen depletions might have been the cause of oyster mortalities on planted beds in Bon Secour Bay although no direct evidence was provided. Lenihan and Peterson (1998) associated mass mortality of oysters with extended periods of anoxia in the Neuse River estuary in North Carolina. Other researchers have reported that oysters can survive several days of hypoxic and/or anoxic conditions (Sparks et al. 1957, Baker and Mann 1992). In the present study, oysters survived anoxia for 3 days in February 1999 but did not survive a 6-day anoxic period in July 1999. There was a difference in the duration of the two anoxic events, but maybe more importantly, there was a difference in water temperature. These findings corroborate those of various authors who found that oyster tolerance to anoxia decreased with increasing temperature (Dunnington 1968, Andrews 1982, Shumway and Koehn 1982, Stickle et al. 1989). Stickle et al. (1989) report LT_{50} (lethal temperature) values of over 28 days for oysters in anoxic conditions at temperatures of 10 °C and only 3 days for oysters at 30 °C. Oysters buried in anoxic sediments survived for more than 5 wk at temperatures less than 5 °C but only 4 days at temperatures greater than 25 °C (Dunnington 1968). Therefore, it appears unlikely that a viable oyster population could be maintained at Fish River Reef due to periodic anoxic events coincident with high water temperatures in the summer.

Temperature and salinity on bottom were consistently similar to temperature and salinity 40 cm above bottom, and ranged from 11.8 to 32.8 °C and 4.4 to 29.8 ppt, respectively. Variations in oxygen concentrations at both depths also follow a similar pattern over time. Although oxygen concentration on bottom was normally lower than oxygen concentration 40 cm above bottom, the differences were small. The lower oxygen tension near bottom was

TABLE 3.

Height (mm) of oysters deployed on bottom and 20 cm and 40 cm above bottom (mean ± SE) at Fish River Reef in 1999

Date	Bottom	20 cm	40 cm
06 Aug. 1999	17.9 ± 0.36	17.9 ± 0.36	17.9 ± 0.36
08 Sep. 1999	27.03 ± 0.93	26.80 ± 0.84	27.25 ± 0.91
27 Sep. 1999	28.40 ± 1.35	30.69 ± 0.99	30.00 ± 1.09
01 Nov. 1999	36.28 ± 1.30	36.56 ± 1.57	38.08 ± 1.26
22 Feb. 2000	44.9 ± 2.4	50.4 ± 2.2	49.6 ± 1.5

probably due to high BOD at the water sediment interface, and low mixing due to boundary layer effects. The difference in oxygen tension at the two depths was more pronounced between October 3 and 6, 1998, most likely due to neap tides and calm winds which did not induce much mixing of the bottom waters shortly after Hurricane Georges had caused the deposition of a lot of sediment. This hypoxic event appears to have temporarily reduced the growth of oysters deployed on bottom and 20 cm above bottom, more than it reduced the growth of oysters 40 cm above bottom. Widdows et al. (1989) and Baker and Mann (1992, 1994), attribute such a reduction in growth during a hypoxic event to a reduced feeding rate. Less than 2 months later, the oysters on bottom had undergone compensatory growth and had caught up with oysters on the 40-cm platforms.

Vertical profiles of the water column suggest periodic stratification due to a pycnocline between 1 m and 2 m depth (Fig. 3; Table 4). The data does not support the presence of a thermocline. Others have reported similar low-oxygen events as a result of pycnoclines in Mobile Bay (May 1972, Turner et al. 1987, Schroeder et al. 1990) as well as in Chesapeake Bay (Breitburg 1990, Sanford et al. 1990). These events are said to occur in stratified waters when high salinity bottom waters are exposed to high BODs in bottom sediments. Vertical mixing, vertical advection, and water mass movements within shallow estuaries such as Mobile Bay are wind driven (Ward 1980, Wiseman et al. 1988). In the absence of a strong unidirectional wind, the water column may stratify creating a hypoxic layer near bottom. In February, July, and August 1999, when the anoxic events in this study were recorded, wind velocities were variable and wind direction changed constantly. The formation of waves is dependent on a unidirectional wind with ample fetch and duration. Wind conditions during these periods were not conducive to the formation of waves large enough to mix the water column. Baker and Mann (1992) and Osman and Abbe (1995) associated hypoxia with a reduction in postlarval growth of *C. virginica*. A slower growth of juvenile oysters prolongs their susceptibility to disease and predation by crabs, drills, and fish (Dittman et al. 1998, Grant 1996). No signs of predation were detected on the oysters deployed outside the bags in the summer of 1998. In June 1999, we noticed some oyster drills on deployed oysters but total mortality of oysters in July prevented documenting potential effects of predation.

TABLE 4.

Presence or absence of a halocline and/or thermocline at Fish River Reef from June through October 1999

	Halocline		Thermocline	
Date	Present	Absent	Present	Absent
2/5/99		+		+
25/5/99		+		+
3/6/99		+		+
8/6/99	+			+
9/6/99	+			+
15/6/99		+		+
16/6/99		+		+
21/6/99	+			+
22/6/99	+			+
30/6/99		+		+
6/7/99	+			+
13/7/99		+		+
21/7/99	+			+
26/7/99		+		+
2/8/99		+		+
11/8/99		+		+
17/8/99		+		+
23/8/99		+		+
31/8/99		+		+
8/9/99		+		+
15/9/99		+		+
24/9/99		+		+
29/9/99		+		+
12/10/99		+		+
19/10/99		+		+
26/10/99		+		+
1/11/99		+		+

The 1-year growth rate of oysters in the present study was 3.25 mm month^{-1}. Anderson (2000) raised oysters in suspended culture in Mobile Bay, approximately 3 km south of FRR and reported growth rates similar to those reported herein. In both studies, oyster growth rates in Bon Secour Bay were less than the 4.65 mm month^{-1} reported by Supan (1983) in Mississippi Sound. Various workers have discussed the importance of factors such as location, timing of larval settlement, genetics and year-to-year environmental variation on growth (Newkirk et al. 1977, Losee 1979, Crosby et al. 1991, Dittman et al. 1998, Bataller et al. 1999). With such variability in oyster growth rates, we believe that growth rate determinations would only be relevant when averaged over several growing seasons.

Traditional methods of estimating oyster growth are by measuring changes in height. Height measurements were not a good indicator of growth in the present study. Walne (1958) and Hilbish (1986) discuss the inadequacy of using linear measurements as a sole indicator of growth in bivalves while Losee (1979) multiplied length and width of oysters and used changes in square area as an index of growth. In the present study, oysters deployed at 40 cm above bottom initially grew faster than oysters deployed on bottom but, within 5 months, the average height of oysters at both depths was similar. However, morphological observations suggest that oysters at 40 cm were more rounded and cup-shaped than those on the bottom. Moreover, data in Table 2 suggest that although the average height of the oysters at 40 cm was shorter than the average height of oysters on bottom, oysters on bottom had less dry meat weight and a smaller average condition index. We propose that oxygen stress and/or siltation stress caused the oysters maintained on bottom to grow into coons. Probably, the silt settled through the oyster bags, and in situations where there were no void spaces under the bags (on bottom), the silt gathered in the bag. The bags on the platforms were lying on shell and the silt was washed down into the shell. Although the oysters were lying horizontally in the bags, and increased height would not allow them to grow out of the sediment, it appears that the reaction to adverse conditions is to grow into coons, regardless of orientation.

Results of the present study suggest that even with restoration efforts such as cultch and oyster deployment, it is unlikely that the relic oyster reef at the Fish River site can be restored to a healthy and productive oyster reef. Moreover, the low oxygen events observed at FRR could be occurring at other relic reefs in Bon Secour Bay, thus the need for investigations similar to the present work before restoration is attempted.

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DWARF MALES IN THE PUELCHE OYSTER (*OSTREA PUELCHANA*, D'ORB.): DIFFERENTIAL MORTALITY OR SELECTIVE SETTLEMENT?

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ABSTRACT *Ostrea puelchana* is the only ostreid for which a "dwarf male" phenomenon has been described. Adult females (>55 mm) "carry" small individuals settled on a expansion of the anterior margin of the concave shell. This phenomenon co-occurs with sex reversal. These small oysters mature as males and have their growth severely retarded as a result of interaction with the carrying female oyster. The non-random distribution of small males is, at least in part, a consequence of the higher survival rate of settlers on the platform of adult females, where they are sheltered from the foraging activities of chitons. In the present work "carriage" was studied in wild oysters from three natural grounds and in farmed oysters. The results show that in farmed oysters, carriage begins at a larger size than in wild oysters, suggesting that the attraction effect exerted by the adult oysters on the larvae ready to settle is related to age rather than to the size of the oyster. Settlement is constrained by the previous occupation of the platform. The settlement and/or survival of new recruits is inversely related to the presence and size of previous recruits. Recruitment on dead and living oysters, and the settlement of *O. puelchana* larvae on adults of *O. puelchana* and *O. edulis* were experimentally assessed. The results of both experiments suggest: (1) the specificity of the carriage phenomenon and (2) the existence of an active selection of the platform by the larvae. Thus, the carriage phenomenon may be explained by two simultaneous mechanisms: the selective mortality of recruits settled on the shells, and the differential larval settlement through a process of active selection.

KEY WORDS: *oysters,* Ostrea puelchana, *dwarf males, settlement, mortality*

INTRODUCTION

The Patagonian flat oyster, *Ostrea puelchana*, is the only ostreid for which a "dwarf male" phenomenon has been described (Calvo and Morriconi 1978, Pascual et al. 1989). Besides sharing the general breeding pattern with the rest of Ostreas species, *O. puelchana* shows a unique system in which larvae setting on a expansion of the internal surface of the anterior margin (the shell platform) of the concave shell of adult females (Fig. 1), mature as male oysters at about 2 mm of shell size. Growth in these males is severely retarded as a result of interaction with the carrying female oyster (Pascual et al. 1989).

During the settlement season oyster larvae settle on all available hard surfaces but preferentially on the shells of living oysters (Pascual and Zampatti 1995). In natural grounds, the number of recruits settled during the settlement peak is significantly higher on living oysters than on dead oysters showing that a chemically mediated adult-larval interaction triggers settlement in this species (Pascual and Zampatti 1995). Larval settlement on live oysters occurs on the outer surface of the shells and on the anterior platform of the concave shell, where they are sheltered by the flat shell. The concentration of small individuals is much higher in the inner surface of the platform than on the external surface of the shells. Calvo and Morriconi (1978) suggested that the high concentration of small individuals on the platform could be due to a localized settlement response triggered by a chemical released by the females.

Most recruits settling on the surface of live oysters die during their first month of life (Pascual 1997). This fact led to an alternative explanation for the non-random distribution of small individuals, namely that settlement is random, but spat that settle on the internal platform have a higher survival rate (Pascual 1997). Experimental work carried out later demonstrated that, in the case of the puelche oyster, grazing by chitons is an important mortality source for oyster recruits that settle on the outer shells of oysters (Pascual 1997). Shelter from chitons may result in higher survival rate of settlers on the shell platform of adult females (Pascual 1997).

Settlement of spat on the outer surface of the shells of live oysters is independent of the oyster's size. This pattern suggests that each oyster attracts, on average, a constant number of larvae (Pascual and Zampatti 1995) and that this effect is maintained during the entire lifespan. Settlement of spat on the internal shell platform, on the other hand, does not occur until the oyster reaches 55 mm in size (Calvo and Morriconi 1978). This unambiguous pattern strongly suggests the existence of a mechanism of active selection by the larvae or an attraction by the female carrier oyster.

In this study, I explore the hypothesis of the existence, in *Ostrea puelchana*, of a differential larval settlement on the platform through a process of active selection, a mechanism that can also explain the *carriage* phenomenon. First, I present novel information concerning the pattern and timing of carriage in wild and farmed oysters, and the spatial arrangement of settlers on the platform of wild oysters in one of the main oyster grounds. Second, I compare larval settlement between oyster species, and between live and dead *Ostrea puelchana*.

MATERIAL AND METHODS

Carriage in Oysters of Three Natural Grounds: Banco Reparo, Las Grutas, and Bajo Oliveira

The oyster ground at Banco Reparo occupies an area of 30 km^2 on the NW of San Matías Gulf, Argentine Patagonia (40°40′S: 63°30′W; Fig. 2). The bottom is sandy and covered partially by pebbles and mollusk shells. Water currents are strong, and depth ranges from 2 to 3 m at low tides. Maximum oyster density in this ground is 3.2 individuals per m^2 (Pascual 1993).

The oyster ground studied at Las Grutas is located in open waters on the NW coast of the gulf, 15 km south of Banco Reparo

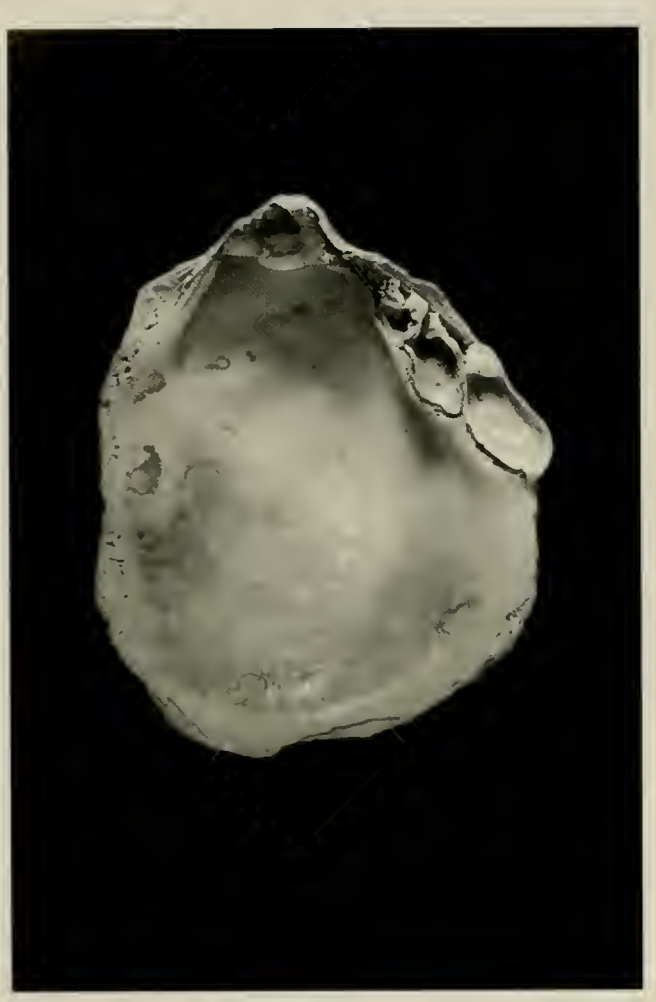

Figure 1. ***Ostrea puelchana*****: female oyster carrying three dwarf males on the anterior margin of its concave shell (shell platform).**

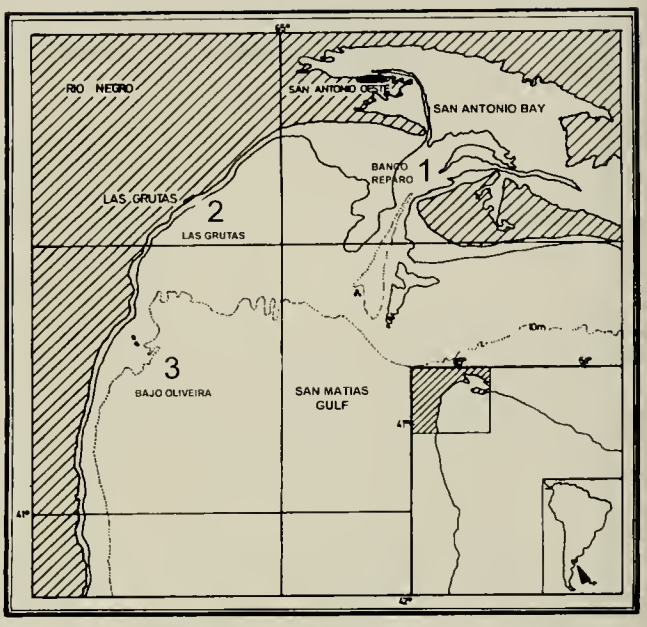

Figure 2. San Matías Gulf: location of the main Puelche oyster grounds. 1 = Banco Reparo; 2 = Las Grutas; 3 = Bajo Oliveira.

(40°48′S; 65°05′W; Fig. 2). The bottom consists of coarse sand and shell, occasionally interrupted by limestone platforms. Tidal currents are weaker than in Banco Reparo and depth ranges from 2.5 to 6 m at low tide. The oyster ground occupies an area of approximately 2 km^2. Maximum densities (22.0 oysters per m^2) are found in sandy channels and depressions (Pascual 1993).

The oyster ground at Bajo Oliveira is the largest of the San Matías Gulf oyster grounds. It is located at the NW of the gulf (40°51′S; 65°05′W; Fig. 2). The bottom is predominantly sandy and flat, covered by pebbles and mollusk shells. The oyster ground occupies an area of 88 km^2 and it lies in north–south direction with depths ranging from 10 to 20 m (Pascual 1993). Maximum oyster densities (0.8 individuals per m^2), recorded in previous surveys, are found in the northwest portion of the ground.

Samples were collected on April 1987 from Banco Reparo and on November 1987 from Las Grutas. At each site, a 100-m transect was drawn across the central densely covered area of the ground. All individuals found in a 2-m wide path along the transect were collected by divers. Oysters were measured (height: maximum distance in mm from the umbo to the opposite margin) and the number and size of dwarf males settled on the shell platform of each carrier oyster was recorded.

At Bajo Oliviera, a survey was performed on May 1988 with a research vessel equipped with a commercial dredge (2.5 m dredge width). Nine hauls (towing time: 10 min; swept area per tow: 2,700 m^2) were performed over the high-density area of the ground (Pascual 1993). One sack of oysters (approx. 40 kg) was randomly chosen from the total catch of each haul. All the oysters were measured (total height), sacrificed and the number and size of dwarf males in the shell platform of each oyster were recorded. Two of the nine samples randomly were chosen for a more detailed sampling. Recruits (spat settled during that season) on the outer shells of oysters were counted and measured under binocular microscope; the oysters were then opened and recruits and dwarf males on the shell platform of oysters were counted and measured.

Carriage in a Stock of Farmed Oysters

The oysters used in this experiment were collected on artificial collectors placed on the oyster ground of Las Grutas during the 1983 through 1984 settlement season. In April 1984, juveniles (mean height = 26.7 mm; SD = 4.3; n = 775) were tranferred for growth-out (Pascual and Bocca 1988) to Banco Garzas, a protected site within San Antonio Bay (Fig. 2). The oysters were placed in plastic mesh bags tied to off-bottom racks, anchored to the bottom. Over a 50- mo period, the oysters were measured on a monthly or bimonthly basis. At each sampling date, 30 individuals were randomly collected. Size increments and spat recruitment on the internal shell platform were recorded.

Selectivity of Larval Settlement

Experiment 1: Settlement of *Ostrea puelchana* larvae on *O. puelchana* and *Ostrea edulis*

This experiment was designed to evaluate the level of specificity of larval settlement on the outer shells and in the shell platform of the puelche oyster. Adult individuals of *Ostrea edulis* (European flat oyster) and *O. puelchana* were exposed to the recruitment of a lot of pediveligers of *O. puelchana*. The experiment was carried out in the hatchery of Ronce-les-Bains (IFREMER,

France) in May 1989. The oysters of both species (n = 12) used in the experiment had similar size (mean height *O. puelchana*: 85.4 mm ± 4.6; mean height *O. edulis*: 85.6 mm ± 4.8). Four sieves (40 × 15 cm) were placed in each of three raceways. Two oysters (one Puelche oyster and one European oyster) were placed in each sieve. During the experiment the oysters were fed with an *Isochrysis galbana* diet and were maintained in an open water circuit. The puelche oyster larvae were produced in the same laboratory. The experiment begun when 50% of the larvae in the larval tanks reached the pediveliger stage. A batch of 50,000 larvae was introduced in each sieve. Oysters were sacrificed at day 4, when free larvae were no longer observed swimming in the sieves. The spats settled on the outer shells and on the shell platform of each oyster were counted and measured using a compound microscope.

Experiment 2: Comparison of Larval Settlement on Dead aod Live Oysters

This experiment was carried out at Las Grutas oyster ground during the 1990 through 1991 settlement season. Oysters from the natural ground were collected, sorted to get a uniform size stock (mean height = 76.5 mm; SD = 4.8; n = 186), cleaned from epibionts with a wire brush, and allocated randomly into two groups (treatments) of 93 individuals each. All oysters in one group were sacrificed, and the empty clean shells were tied together with a plastic band. Each treatment was arranged in three replicates of 31 oysters, each placed in a separate culture lantern. Mesh size guarantied predator exclusion. The six lanterns were suspended from a long line, alternating treatments. The structures remained suspended at mid water during the entire settlement season (December 28, 1990 to April 22, 1991).

At the end of the season, the number and size of the recruits settled on the outer shells and shell platform of each oyster (live or dead) were recorded. Carriage was expressed as the proportion of recruits settled on the platform in relation to the total number of recruits settled per "substratum oyster." Data were arc-sine transformed for analysis. The number of recruits settled on the shell platform and outer shells in live and dead oysters was statistically analyzed with a Nested Anova test (Sokal and Rohlf 1969).

RESULTS

Carriage in Oysters of Three Natural Grounds: Banco Reparo, Las Grutas and Bajo Oliveira

Samples obtained in Banco Reparo and Las Grutas enabled us to estimate the full size structure of the population. Figure 3 shows the size frequency distribution of the whole population (free oysters and dwarf males), in the two sites. These size distributions are bimodal, one mode representing the dwarf males settled on the shell platform of carrier oysters (mean = 11.7; SD = 6.9; n = 263, for Banco Reparo, and, mean = 8.2 mm, SD = 6.3; n = 418 for Las Grutas), and the second representing free oysters (mean = 74.1 mm; SD = 13.6; n = 597 for Banco Reparo and mean = 66.7 mm, SD = 18.7; n = 722 for Las Grutas).

Carriage of small males begins at a size of 55 mm at Banco Reparo, and at 50 mm at Las Grutas. In both grounds, the percentage of carriage (number of carrier oysters/total number of oysters larger that 50 mm × 100) in the population increases as the size of the oyster increases (Fig. 4). Carrier oysters in both grounds hold a maximum of six epibiotic males per carrier oyster, 61% of

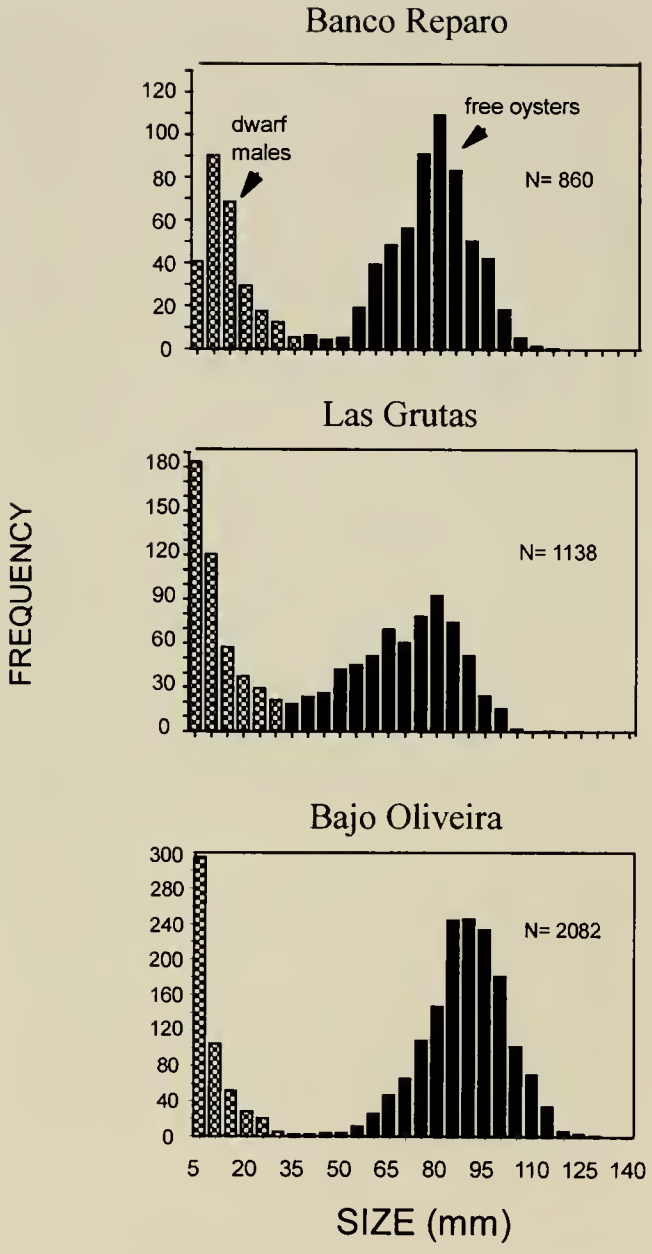

Figure 3. Size frequency distributions of the complete oyster population, composed by free oysters (carrying or not carrying dwarf males), aod dwarf males fixed on the shell platform of carrier oysters. Graphs represent the oyster populations of Banco Reparo (above), Las Grutas (center), and Bajo Oliveira (below).

the carriers holding one epibiotic male, in Banco Reparo, and 68% in Las Grutas.

The oyster population of Bajo Oliveira is composed of large individuals (Fig. 3). The population of free oysters has a mean size of 89 mm (SD = 13.4; n = 1550). The complete population, including dwarf males, is clearly bimodal. The mode representing dwarf males has a mean size of 9.1 mm (SD = 6.5; n = 532).

The percentage of carriage is very high in this ground, an 88.28% of the oysters larger than 50 mm carry dwarf males on the platform. The oysters carry a maximal number of five dwarf males per carrier oyster. Of the carrier oysters, 63% carry two or more dwarf males. A detailed observation of new settlers (recruits of the

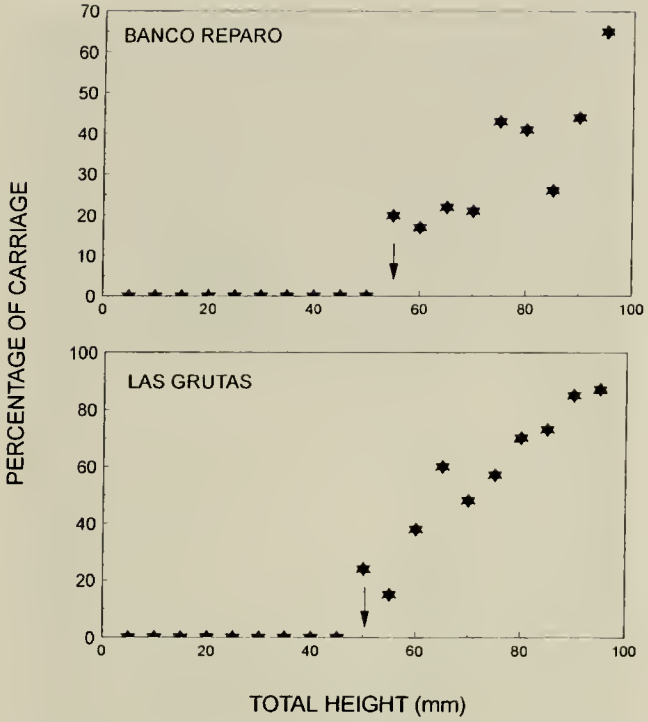

Figure 4. Percentage of carriage (proportion of carrier oysters/total number of oysters >50 mm × 100) in relation to the oyster's size at the oyster grounds of Banco Reparo (above) and Las Grutas (below).

season) showed that oysters had a very low recruitment on the shells, while recruitment on the platform was very high. A maximum of 52 settlers fixed on the platform of carrier oysters was recorded (Fig. 5).

Oysters settled on the platform were divided into dwarf males (age >1 y) and newly settled spat (recruits of the last season). Figure 6 shows the relationship among the number of spats newly settled (recruits of the last season) on the shell platform and the size of previously settled dwarf males (weighted by its number). The number of new settlers decreases as the size of the previously settled dwarf males increases.

Carriage in a Stock of Farmed Oysters

Recruitment on the platform was detected when the experimental lot reached a mean size of 70.6 mm (SD = 7.9; n = 164) and an age of 48 mo. Carrier oysters in the lot represented a 23% of the total stock and were in a size range of 65 to 82 mm (mean height = 73.3 mm; SD = 8; n = 38).

Selectivity of Larval Settlement

Experiment 1: Settlement of Ostrea puelchana *larvae on* O. puelchana *and* Ostrea edulis

Mean settlement on the shell platform of the puelche oysters was, at the end of the experiment, 1.66 spat per oyster (Table 1). The European flat oysters did not show settlement along the anterior margin.

Settlement on the shells was heavier in the European oysters (Table 1) even when non-significant differences were detected (t = 1.42; $P > .05$). The size of recruits, settled on both species, did not show differences among species.

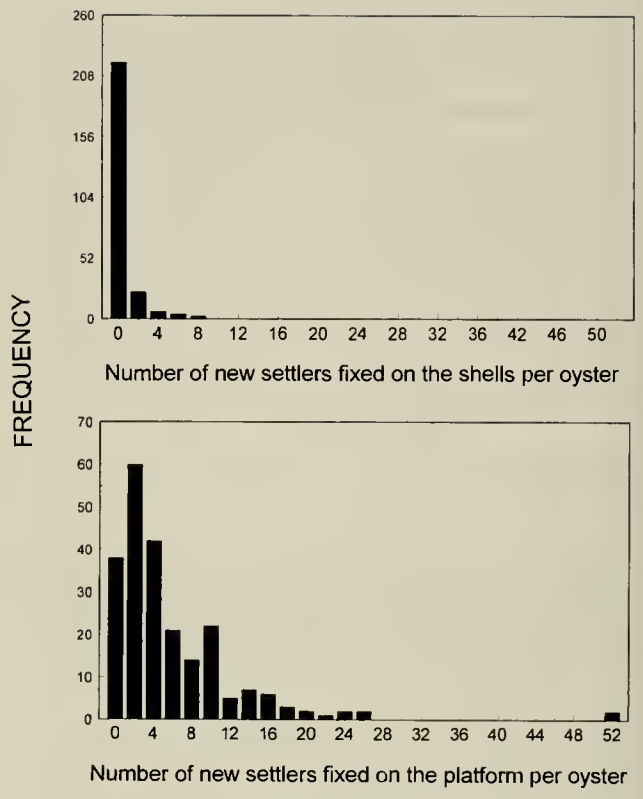

Figure 5. Settlement of spat on living oysters during the 1988 settlement season at the oyster ground of Bajo Oliveira. Graphs show the number of spat newly settled on the outer shells per oyster (above), and the number of spat newly settled on the shell platform per oyster (below).

Experiment 2: Comparison of Larval Settlement on Dead and Living Oysters

Larvae did settle on the outer shells and shell platform of oysters assigned to both treatments and replicates (Table 2). However, settlement on the shells of dead oysters was significantly lower than on the shells of living oysters (Nested Anova: F = 59.85; P

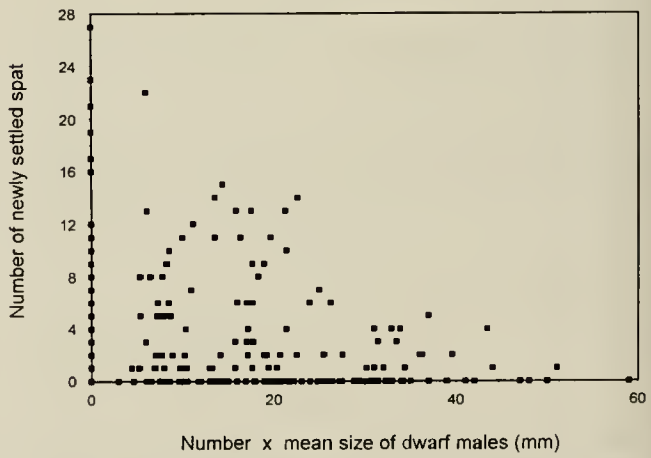

Figure 6. Pattern of settlement of spat on the shell platform of oysters of the ground at Bajo Oliveira. Relationship among the number of newly settled spat (recruits of the last season) and the size of dwarf males previously settled (weighted by its number), per oyster.

TABLE 1.

Results of a experiment designed to evaluate the level of specificity of larval settlement on the outer shells and the shell platform of the puelche oyster.

	O. puelchana	*O. edulis*
No. of spat on outer shells per oyster		
Mean ± SD	268.5 ± 135	347.5 ± 136.4
n	12	12
No. of spat on shell platform per oyster		
Mean ± SD	1.7 ± 1.1	0
n	12	12
Size of spat		
Mean height (in mm) ± SD	2.5 ± 1.4	2.8 ± 1.3
n	324	359

Adult individuals of *O. puelchana* and *O. edulis* were exposed to the recruitment of a batch of pediveligers of *Ostrea puelchana*.
SD = standard deviation.

< .005). In addition, the number of recruits settled on the platform was significantly higher in live oysters (Nested Anova: $F = 32.16$; $P < .005$).

The recruits which settled on living oysters reached a greater mean size (8.7 mm; SD = 3.9; n = 303) than those settled on dead oysters (6.6 mm; SD = 3.7; n = 176) ($t = 11.22$; $P < .005$).

DISCUSSION

The probability of carrying dwarf males on the shell platform increases as the size of the female oyster increases. From the time the oyster reaches a size of 50–55 mm, the size at which carriage begins, the shell platform gradually widens. The epibiotic male's own growth additionally produces a hollowing in the platform, which (once the small male detaches) becomes more favorable for the settlement of new recruits (Pascual 1993). This correlation between the development of the shell platform and the oyster's size is reflected in the number of dwarf males carried per oyster: in Banco Reparo and Las Grutas, 61% and 68%, respectively, of the carrier oysters carry 1 dwarf male, while in Bajo Oliveira, where the mean population size is higher, 63% of the oysters carry two or more dwarf males.

The pattern of recruitment on the shell platform analyzed in oysters of Bajo Oliveira, suggests that settlement is constrained by the previous occupation of the platform. The settlement and/or survival of new recruits are inversely related to the presence and size of previous recruits.

TABLE 2.

Results of a experiment carried to evaluate larval recruitment on living and dead oysters.

Treatment	Lantern	n	No. spats on outer shells (mean ± SD)	No. spat on shell platform (mean ± SD)
Living oysters	1	28	3.10 ± 2.43	2.44 ± 1.69
	2	28	3.60 ± 2.96	1.88 ± 1.70
	3	31	4.06 ± 2.15	2.12 ± 1.78
Dead oysters	1	29	1.72 ± 1.55	0.64 ± 1.00
	2	29	2.10 ± 1.89	0.82 ± 0.64
	3	29	2.62 ± 2.05	0.62 ± 0.55

Values are mean numbers of recruits settled on the outer shells or shell platform.
SD = standard deviation.

Post-settlement mortality is very high during the 3 mo following recruitment and similar among grounds (Pascual 1997). As it was demonstrated previously, grazing (or bulldozing) by epibiotic chitons is an important mortality source for oyster recruits that settle on oysters (Pascual 1997). The low recruitment on shells at Bajo Oliveira, together with the intense settlement on the platform, suggests that those differential mortality agents indeed operated on recruits.

In natural grounds, carriage is a character that is expressed unambiguously once the oyster exceeds 50 mm in size, strongly matching the size of sex reversal to the female phase in normal conditions (Calvo and Morriconi 1979). The debut of "carriage" in farmed oysters took place at a mean size 15 mm higher than that of wild oysters of the same locality, suggesting that the attraction effect could be related to age rather than to size of the oyster.

The results obtained from experimentally comparing recruitment on dead and living oysters confirm those reached while evaluating the recruitment in natural grounds (Pascual and Zampatti 1995) and indicate that settlement on the platform is a response to an active attraction effect produced by the live oyster and not merely a result of a random recruitment process.

The specificity of the carriage phenomenon was proved by experimentally evaluating the inability of the European oyster, *O. edulis*, for "carrying" epibiotic oysters on the platform. The specificity of the carrying phenomenon is supported by the fact that recruits of the congeneric *O. spreta*, which co-occurs with *O. puelchana* in Patagonian waters, were never observed settled on the platform of puelche oysters or "carrying" epibiotic oysters themselves.

The high density of spat settled on the outer shells of both Puelche and European oysters could be explained by the large number of larvae offered in the experiment. In contrast, the relatively low number of spat settled on the shell platform of puelche oysters may be related to the reproductive stage of the oysters used in the experiment, since there is some evidence suggesting that the attraction effect exerted on the larvae ready to settle is weaker in already spawned females (Pascual, unpublished). However, this topic is currently under investigation.

Calvo and Morriconi (1978) suggested the existence of a chemical attractant triggering a settlement response of the larvae that settle on the shell platform of adult oysters. The match between the initiation of the female phase (sex reversal) and "carriage" (Morriconi and Calvo 1978) suggests a relationship between this phenomenon and the reproductive cycle. A chemical linked to gonadal maturation may trigger the differential settlement of larvae on the platform. Alternatively, increased carriage could be the result of the development of the platform, which enlarges with the age of the female oyster, as well as of the hollowing of the platform by the growth of dwarf males. The big platform of old oysters from Bajo Oliveira, which carried dwarf males of maximal sizes, is consistent with this scenario.

The development and adaptive significance of the carriage of dwarf males by females of the puelche oyster can be conceptualized from three viewpoints.

Functional. Fertilization occurs in two alternative ways in this species (via free males or dwarf males). The functionality of carriage is based on two features of this mechanism: the architecture of the relation female carrier-carried male, and the syn-

chronization in gonadal maturation in both partners. The result is an increase in fitness for both sexes (Calvo and Morriconi 1978, Pascual et al. 1989).

Developmental. Male dwarfness is a result of retarded growth rate of males by the interaction with a female carrier (Pascual et al. 1989). As a result, epibiotic males that remain attached to the platform have a long lifespan, with lowered predation risks and less sperm loss.

Topological. The relatively high concentration of individuals on the platform of oysters >50 mm, as compared to the surface of the shells can be explained by two mechanisms: selective mortality of recruits and active selection of larvae.

The non-random distribution of small males is, at least in part, a consequence of the higher survival rate of settlers on the shell platform of adult females, where they are sheltered from the foraging activities of epibiotic chitons (Pascual 1997). This ecological contingency may have provided a selection pressure favoring development of selective settlement behavior.

Larval attraction by adults is a process already described for *O. puelchana* (Pascual and Zampatti 1995) and other oyster species (Crisp 1967, Veitch and Hidu 1971, Bonar et al. 1990). Active selection of the platform by the larvae could be regarded as a special case of the general process of attraction that adults exert over larvae ready to settle. However, the fact that carriage occurs only when the oyster exceeds 50 mm in size, while attraction to the larvae ready to settle is independent of the oyster's size (or age), suggests the existence of two different mechanisms.

Summing up, the carriage phenomenon may be explained by two simultaneous processes: differential survival of the recruits settled on the platform, which provides a refuge against grazing, and the differential larval settlement through a process of active selection.

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COLLECTION OF PEARL OYSTER (FAMILY PTERIIDAE) SPAT AT ORPHEUS ISLAND, GREAT BARRIER REEF (AUSTRALIA)

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ABSTRACT The aim of this study was to identify the pearl oyster species recruiting to spat collectors (50% shade mesh in polyethylene mesh bags) and to examine temporal and depth differences in recruitment at Pioneer Bay Orpheus Island, Great Barrier Reef, Australia. Over 24,900 hivalve spat were counted during the 12 months of study (March 1995 to March 1996). In excess of 21,600 pearl oyster spat were collected from three genera of the family Pteriidae: *Pinctada*, *Pteria*, and *Electroma*. The majority of this total (20,378) was the non-commercial species *Electroma papillionacea* (Lamarck 1819). However, collection of eight species of *Pinctada* (2,628 spat) and five species of *Pteria* (approximately 1,200 spat) included species presently used in commercial pearl culture operations throughout the Indo Pacific, notably *Pinctada margaritifera*, *Pinctada fucata*, *Pinctada albina*, *Pinctada maxima*, and *Pteria penguin*. All species collected showed seasonal variation in recruitment intensity. Some species showed brief and temporally isolated types of spawning and associated recruitment pulse of temperate species, whereas the majority displayed peaks in recruitment intensity. A total of 231 *P. margaritifera* spat were collected during the study, with the recruitment peak (mean density 7.1 ± 1.1 spat per collector unit) recorded between May and June 1995. No significant difference in recruitment densities between 2 m and 6 m were recorded for *P. margaritifera*. *Pt. penguin* settled at the highest densities at 6 m in March/April 1995 (mean recruitment per collector ± SE 46.2 ± 6.7). A total of 1,000 *Pt. penguin* spat were collected during the study, and a significant proportion of these settled on the outer mesh bag of the spat collector.

KEY WORDS: Pearl oyster, spat collector, *Pteriidae*, *Pinctada*, *Pteria*, *Electroma*

INTRODUCTION

Pearl oysters from the family Pteriidae are exploited in various cultured pearl production ventures throughout the world. The four major species used for cultured pearl production are *Pinctada maxima* (Australasia), *Pinctada fucata* (Japan, China, India, and Sri Lanka), *Pinctada margaritifera* (French Polynesia, Cook Islands, and Australia), and *Pteria penguin* (Japan, Thailand, Tonga, and Australia).

Although hatchery methods for the production of pearl oysters have been developed over recent years (Gervis and Sims 1992, Southgate and Beer 1997), collection of spat from the wild still has an important role in pearl culture operations. As a relatively low cost and low technology method, wild spat collection may provide spat for developing farming operations (Friedman and Bell 1996), for established pearl culture industries (Coeroli et al. 1984, Sims 1992), and for stock assessment or to estimate species diversity (Knuckey 1995). Spat collection also provides a method of collecting small fragile and cryptic species that are otherwise unobtainable. Reseeding and stock enhancement programmes (Hortle and Cropp 1987, Robins-Troeger and Dredge, 1993, Saudeco et al. 1994) also may be based on spat collection.

Spat collection can be a very effective way to accumulate pearl oyster stock (Crossland 1957); however, efficient spat collection requires information on seasonal patterns of spat recruitment. Recruitment variability, or failure to collect spat, has been identified as a risk that could have high economic costs for pearl culture industries reliant on wild spat collection (Rose and Baker 1994); however, assessment of temporal and spatial trends in spatfall may reduce these risks. To maximize collection of bivalve spat, localized differences in spawning activity (Tranter 1958a, Tranter 1958b, Tranter 1958c, Tranter 1958d, Tranter 1958e, Rose et al. 1990), substrate material preferences (Scoones 1990, Friedman and Bell 1996), the effects of spat collector position in the water column (Robins-Troeger and Dredge 1993), and location (Friedman and Bell 1999) must be recognized and exploited.

Spawning in bivalves is often associated with temperature extremes or other environmental changes (Quayle and Newkirk 1989). Species from temperate regions generally exhibit more discrete and regular spawning seasons and generalizations applied to bivalve reproductive cycles from temperate regions are not always applicable to tropical species (Gervis and Sims 1992). Reproductive seasonality in pearl oysters was described by Tranter (1958c) as "relative breeding intensities" with "major breeding season(s)." In many species of pearl oysters, spawning and subsequent spatfall may occur more than once in the year (Tranter 1958a, Tranter 1958b, Tranter 1958c, Tranter 1958d, Tranter 1958e, Wada et al. 1995, Behazi et al. 1997).

There are few published data on the collection of pearl oyster spat in Australia. The primary objectives of this study were: (1) to identify the species of pearl oysters that recruited onto spat collectors in Pioneer Bay, Orpheus Island; (2) to determine the seasonal trends in recruitment and; (3) to determine the effect of spat collector depth on the density of recruitment.

MATERIALS AND METHODS

Location of Study Site

Orpheus Island (18°35′S, 146°29′E) is within the Palm Island group of high continental islands approximately 80 km north of Townsville and approximately 20 km offshore (Fig. 1). Pioneer Bay is on the leeward (western) side of Orpheus Island (Fig. 1). The surface longline used in this study was located at the northern

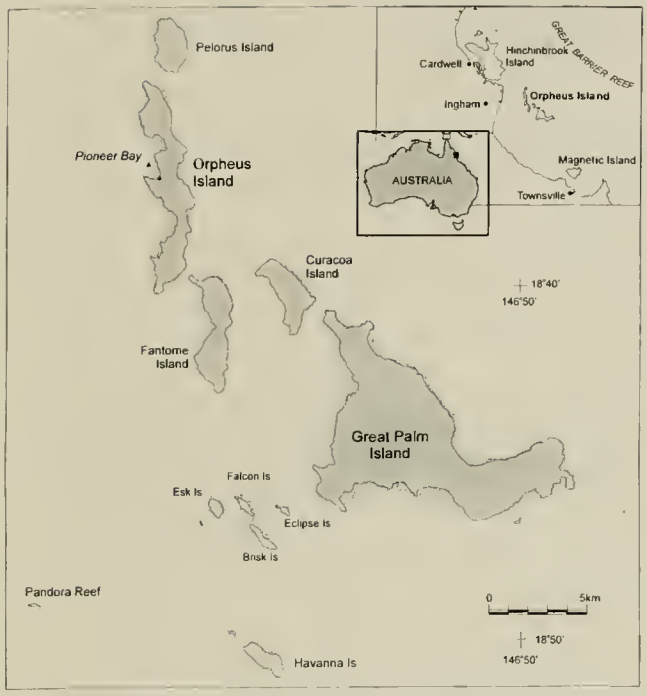

Figure 1. Location of Orpheus Island spat collection site within the Palm Islands, 20 km offshore and 80 km from Townsville, north Queensland. The surface longline (▲) is 50 m from the reef flat in 10–13 m deep water and adjacent to James Cook University's Orpheus Island Research Station (●).

end of Pioneer Bay, approximately 50 m from the reef flat with a depth under the longline of 10–13 m. This study began in March 1995 and concluded in March 1996.

Collection of Spat

Ten spat collectors were deployed on the longline, five at each depth of 2 m and 6 m. Collectors were attached to a 7-m length of rope, weighted at the end. Each spat collector consisted of two 40 × 70 cm mesh bags (polyethylene mesh 1.5 mm fiber size, 8 × 5 mm mesh size) with three 0.5 m^2 sheets of 50% shade mesh (0.5-mm fiber size, 2 × 1-mm mesh size). The collection material (shade cloth and mesh bag combination) was chosen because of availability, low cost, durability, re-useability, and successful use of these materials in hatchery trials with pearl oysters (Southgate and Beer 1997).

Collectors were changed every 4 weeks, at which time they were washed with high-pressure seawater to remove sediment and loose spat. Both shade mesh and mesh bags were inspected manually and spat removed with a scalpel. The water used to wash the substrates was sieved through 3-mm and 1.7-mm mesh screens. Spat caught on the 1.7-mm screen were preserved in 10% buffered formalin for later counting and identification using a stereo-dissector microscope. Spat were identified using keys (Hynd 1955, Takemura & Okutani 1955, Takemura & Okutani 1958), information from previous studies (Shirai 1994, Lamprell and Healy 1998). Validation of species identification was obtained from juveniles grown out from collected spat (unpublished data).

Analysis

Recruitment data for each species were analyzed with univariate, two-factor ANOVA examining the factors of depth and time. Monthly recruitment data required $\log_{10}$ (×+1) transformation and was examined with Cochran's test for homogeneity of variance (Zar 1996). Recruitment data for visible and sieved portions were pooled. From the family Pteriidae, the following taxa were counted: *Pinctada margaritifera*, *P. fucata*, *P. albina*, *Pteria penguin*, and *Electroma papillionacea*.

Temperature

Temperature was recorded at 2 and 6 m throughout the study and the maximum, minimum, and mean seawater temperature recorded in Pioneer Bay. Figure 2 shows the changes in sea water temperature with a mean summer maximum of 28.5 °C (March 1995) and a rapid decline to the winter mean minimum of 21.7 °C (August 1995) returning to 29.4 °C in summer (February 1996).

RESULTS

Species Present at Pioneer Bay, Orpheus Island

A total of 20 species of pearl oysters from three genera (*Pinctada*, *Pteria*, and *Electroma*) were recorded in this study. Table 1 presents a summary of the recorded recruitment peaks for the major species during this study. Eight species of *Pinctada* were recorded from a total of 3,972 spat. *P. margaritifera* (Linnaeus 1758), *P. fucata* (Gould 1850), and *P. albina* (Reeve 1857) accounted for 27% of the collected spat; however, *P. maculata* (Gould 1850), *P. maxima* (Jameson 1901), *P. chemnitzii* (Phillipi 1849), and *P. nigra* (Gould 1850) also formed 4% of the spat collected. Unidentified *Pinctada* spat accounted for 69% of the total. Eight species of *Pteria* were recorded during the study. *Pt. penguin* (Roding 1798) was the most common (1,000 spat, 83.3% of genus total), however, *Pt. cypesyllus* (Dunker 1872), *Pt. falcata* (Lamarck 1819), *Pt. coturnix* (Dunker 1872), *Pt. lata* (Gray 1845), *Pt. bernhardi* (Iredale 1939), *Pt. cooki* (Lamprell and Healy 1997), and *Pt. chinensis* (Leach 1814) were also collected. The total collection for the genus exceeded 1,200 spat.

Electroma is a widespread, common, but little known genus within the Pteriidae. At Orpheus Island, four species were present: *E. alacorvi* (Diliwyn 1817), *E. malleus* (Reeve 1857), *E. zebra* (Reeve 1857), and *E. papillionacea* (Lamarck 1819). The former three species were present in very low numbers contrasting with *E. papillionacea*.

Recruitment

Total *Pinctada* Recruitment

Between March 1995 and March 1996, the mean (± SE) number of *Pinctada* sp. spat per collector ranged from 1.3 ± 0.4 in

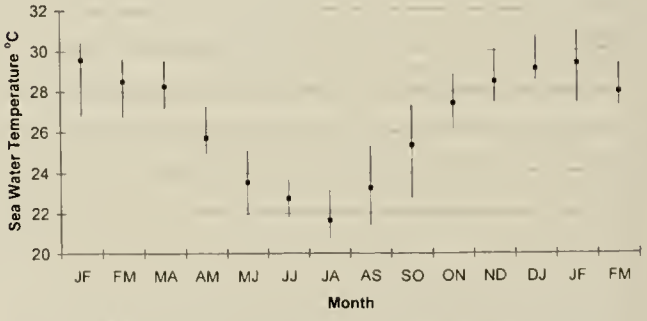

Figure 2. Mean, maximum, and minimum seawater temperature recorded in Pioneer Bay between March 1995 and March 1996.

TABLE 1.

Summary of pearl oyster spat collection at Orpheus Island between March 1995 and March 1996.

Species	Peak Settlement Time	Depth Effect and 'Preference'	Maximum Mean (± SE) Settlement per Collector	Total Number (and % of total) Collected at Peak Settlement	Total Collected March 1995–March 1996
Pinctada margaritifera	May–June	No	7.1 ± 1.1	71 (30.7%)	231
P. fucata	Feb–March	–	–	324 (39.5%)	820
P. albina	Jan–Feb	–	–	132 (45.0%)	293
Pinctada sp.	Feb–March	–	–	1570 (59.7%)	2628
Pteria penguin	March–April	Yes 6 m	46 ± 6.7	462 (46.2%)	1,000
Electroma papillionacea	Oct–Nov	Yes 6 m	335.7 ± 38.4	3357 (16.5%)	20,378

September/October 1995 to 194.5 ± 10.3 in February/March 1996. Figure 3 shows the monthly combined totals for the major *Pinctada* taxa. *P. fucata* was the most common commercial *Pinctada* species recruiting to collectors with a total of 820 spat collected between November 1995 and March 1996. *P. albina* was present in all months except May–June and September–October. Although there was recruitment throughout the year, the species composition recorded each month varied considerably.

A high proportion (69%) of the overall collection totals were species of *Pinctada* that were unable to be positively identified when collectors were examined after 4 weeks deployment. A total of 2,628 unidentified *Pinctada* spat recruited to collectors and were present in all months with the greatest mean (± SE) recruitment in February/March 1996 of 157 ± 9.7 per collector.

Pinctada margaritifera

P. margaritifera showed distinctly bimodal recruitment with a significant difference in densities through time ($P < 0.001$) (Fig. 4). A total of 231 *P. margaritifera* spat were collected with the maximum in May/June (7.1 ± 1.1 per collector unit). Depth did not have a significant effect on recruitment density, primarily due to the low recruitment density and variability in most months. However, in May–June when recruitment was highest and spat were recorded in all collectors, mean recruitment at 2 m and 6 m was 9.8 ± 0.5 and 4.4 ± 0.7 spat per collector, respectively.

Pteria penguin

Recruitment of *Pt. penguin* showed clear seasonal and spatial trends. Mean recruitment per collector was highest between March–April (46.2 ± 6.7) and April/May (31.8 ± 6.3) (Fig. 5), coinciding with a decline in seawater temperature. A total of 1,000 spat were collected with 78% recruiting to collectors between March and May. Recruitment was significantly higher at 6 m ($P < 0.001$) with a total of 702 at an overall mean yield of 11.7 ± 2.7 spat per collector. At 2 m, a total of 298 spat were collected at 4.96 ± 1.1 per collector. Many *Pt. penguin* spat were recorded on the outer surface of collectors during months of high recruitment. Two-thirds of the spat that settled at 6 m were attached to the outer mesh bag.

Electroma papillionacea

Throughout the study, *E. papillionacea* was the most abundant species with a total of 20,378 spat collected. There was a significant difference between the density of recruitment at 2 m and 6 m ($P < 0.001$) with 9,243 spat recorded at 2 m and 11,135 at 6 m. Recruitment between months was significantly different ($P < 0.001$) with peak recruitment in October/November (335.7 ± 38.4 per collector) (Fig. 6) and a maximum density in one collector of 843 spat. This was the highest number of spat per collector of any bivalve species during the study.

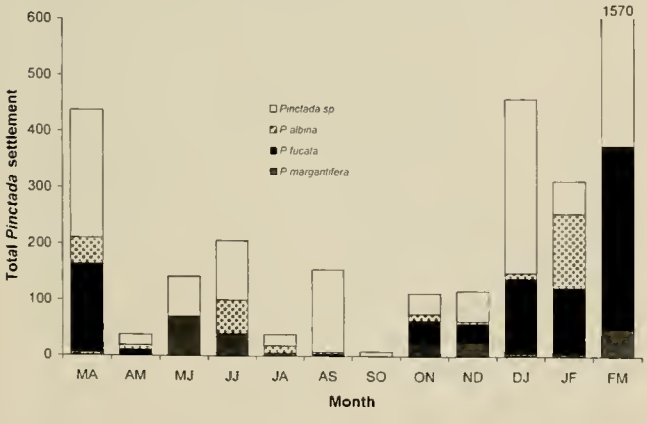

Figure 3. Total monthly recruitment of *Pinctada sp.*, *P. fucata*, *P. albina*, and *P. margaritifera* to spat collectors in Pioneer Bay, Orpheus Island, between March 1995 and March 1996.

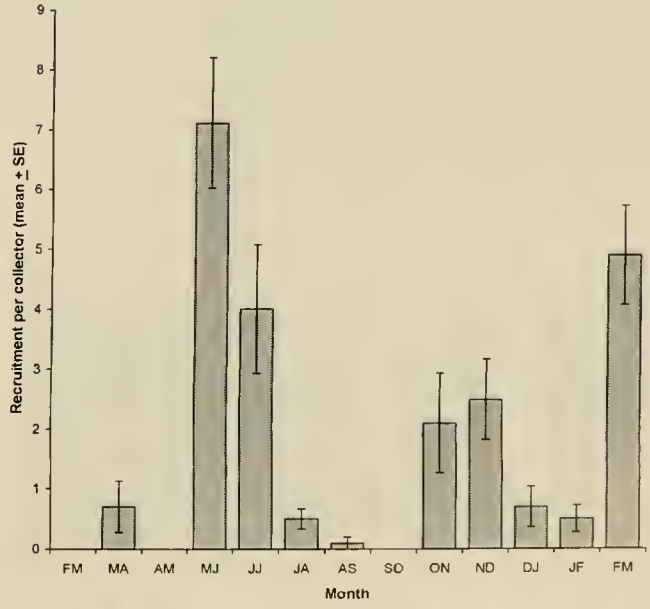

Figure 4. Mean *P. margaritifera* spat recruitment per collector (± SE), between March 1995 and March 1996.

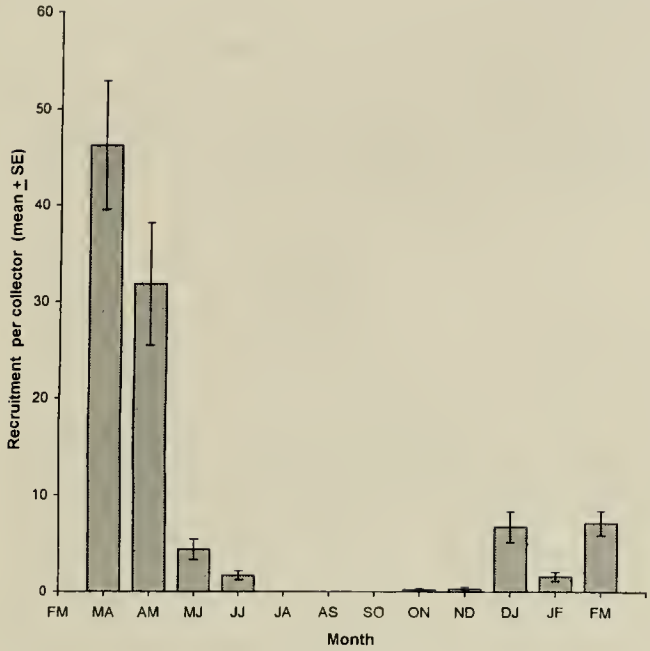

Figure 5. Mean *Pt. penguin* spat recruitment per collector (± SE), between March 1995 and March 1996.

DISCUSSION

This study recorded a relatively high species diversity of Pteriids in Pioneer Bay, Orpheus Island. Eight of the nine species of *Pinctada* recorded in Australian waters (Lamprell and Healy 1998) were collected in this study. Similarly, 13 species within the genus *Pteria* occur in mainland Australia, of which 12 are tropical and 8 were collected during this study. All five *Electroma* species recorded from north Queensland are present at Orpheus Island. Species diversity was considerably higher in this study than in similar studies in northern Australia. For example, spat collection in the Northern Territory recorded nine species of Pteriids (four *Pinctada*, three *Pteria*, and two *Electroma*) (Knuckey 1995), and a similar study on the Great Barrier Reef (Dayton et al. 1989) recorded seven species of Pteriids (seven *Pinctada* and one *Pteria*). Pearl oyster species diversity recorded from spat collection from locations such as the Solomon Islands (Friedman and Bell 1996) and the Cook Islands (R. Braley, personal communication) is considerably lower than that recorded in this study.

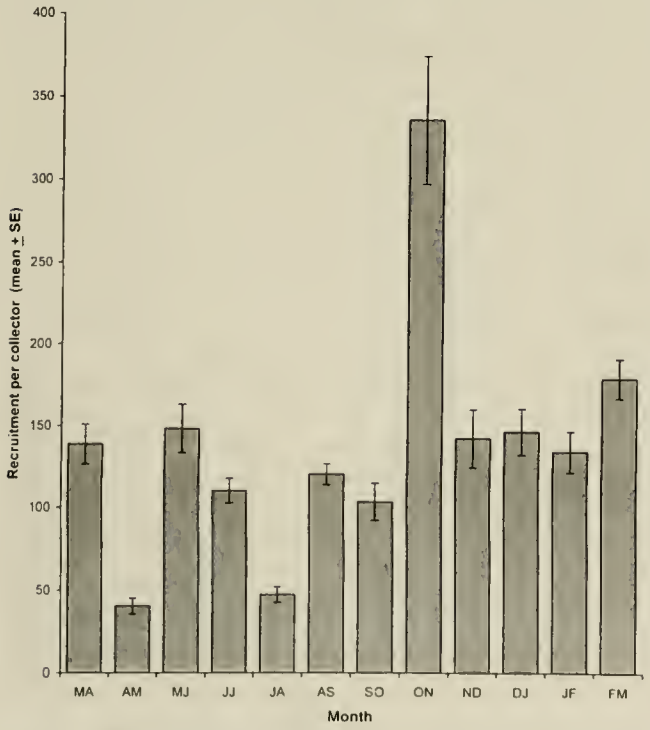

Figure 6. Mean *E. papillionacea* spat recruitment per collector (± SE), between March 1995 and March 1996.

Patterns of spat recruitment over the course of this study challenge conventional wisdom relating to the recruitment of bivalves in the tropics. Recruitment peaks and clearly defined seasons demonstrated by some species (e.g., *Pt. penguin*) contrast with the idea of relatively continuous spawning throughout the year for tropical bivalves (Gervis and Sims 1992). Seasonal variation in seawater temperature is sufficient to suggest that there are significant differences between summer and winter and wet and dry seasons. Therefore, it would be reasonable to assume that discrete and regular spawning seasons should exist for some taxa. However, this study also showed recruitment throughout the year for species such as *E. papillionacea* and *P. margaritifera*, with seasonal peaks, supporting the observation of "relative breeding intensities" (Tranter 1958c).

The bimodal spat recruitment of *P margaritifera* reported in this study corresponds with the earlier work in Australia by Tranter (1958d), who reported spawning activity in two distinct cycles; in the spring–summer (September to February) and autumn–winter (March to August). Highest recruitment of *P. margaritifera* in Pioneer Bay during May to July corresponds with the secondary spawning season in the winter reported by Tranter (1958d). During this study, winter recruitment provided the most *P. margaritifera* spat per collectors; however, more spat could be collected over the summer months as the recruitment period is sustained. Subsequent observations at Orpheus Island showed that winter spatfall is more intense than in summer, but of shorter duration (8–10 weeks).

Spat collection of *P. margaritifera* has been the main source of oysters for culture operations in the Red Sea (Crossland 1957), French Polynesia (Coeroli et al. 1984), and islands in the south Pacific (Gervis and Sims 1992). In French Polynesia (14 °S), Coeroli et al. (1984) reported year-round spat collection with peak recruitment between August and October coinciding with the winter low in sea water temperature (26 to 27 °C). Bimodal recruitment with spring maximum and lower summer recruitment recorded in the Solomon Islands (Friedman et al. 1998, Friedman and Bell 1999), is affected by seasonal changes to water temperature and the monsoonal wet/dry season cycle. *P.margaritifera* spat collection in Donogab Bay on the Sudanese coast of the Red Sea (21°N) was an outstanding success with annual collection of 4.5 million spat in 1921. However, *P. margaritifera* spawning in the Red Sea was restricted to an annual summer event with "no trace of gonad in the autumn or winter" (Crossland 1957). The study by Crossland (1957) showed that spat collection can be extremely effective as a seed collection method, provided accurate seasonal patterns of reproductive activity are known.

Recruitment of other species of *Pinctada* with commercial value (*P. albina* and *P. fucata*) followed the seasonal trends found in Torres Strait, Northern Australia (Tranter 1958b, Tranter 1958c, Tranter 1958e). The total number of *P. albina* spat collected in

Pioneer Bay was low (293) and varied from zero in May–June to a maximum in January–February of 132. Although *P. albina* recruited throughout the year, gonad staging and spat collection in the Torres Strait area (10°S) of Australia, demonstrated "relative breeding seasons" where the heaviest spatfall occurred between June and August during the annual water temperature minima (Tranter 1958b).

Previous studies on reproduction in *P. fucata* in Australia (Tranter 1958e, Sumpton *et al.* 1990), reported two spawning periods within the main breeding season of December to May. The peaks in reproductive activity were termed the "summer spawning" in January–February and "autumn spawning" in April–May; the latter was the more consistent of the two. Similar bimodal reproductive activity has been reported for *P. fucata* in the Persian Gulf (Behzadi et al. 1997). However, spatfall of *P. fucata* has been recorded year round in Northern Australia with highest spatfall occurring between January and March (Tranter 1958e). Early summer recruitment has been recorded for *P. fucata* populations in subtropical Australia (Sumpton *et al.* 1990) with up to 142 spat per collector. This is similar to the pattern of *P. fucata* recruitment observed in this study and these data indicate a broad spawning season from November to March for *P. fucata* in north Queensland. The reproductive seasonality of *P. fucata* varies with latitude and populations from different genetic stocks (Wada 1995, Behzadi et al. 1997). Although *P. fucata* provides approximately 70% of world cultured pearl production (Shokita et al. 1991), spat collection and culture of *P. fucata* is not commercially exploited in Australia at present.

The number of *P. maxima* spat collected during this study was very low. This contrasts the heavy *P. maxima* spatfall reported at Pandora Reef (Fig. 1) by Dayton et al. (1989). This is surprising given the proximity of Pandora Reef to Orpheus Island (12 nautical miles); however, there are differences in turbidity between these two locations and the inshore habitat at Pandora Reef may be more suitable to *P. maxima* (Yukihira et al. 1999). Four years of subsequent observations showed continued low recruitment of *P. maxima* spat in Pioneer Bay (A. Beer, unpublished data 1999).

Differential recruitment at 2 m and 6 m was more clearly shown by *Pt. penguin* than any other species collected. Further research has found this spatial pattern consistent over several spat collection seasons, the majority of spat collected at 6 to 8 m (A. Beer, unpublished data 1999). Numbers of *Pt. penguin* spat were greater on the outside of the collectors, behavior previously recorded for *Pt. penguin* (Gervis and Sims 1992). *Pt. penguin* at Orpheus Island generally settle and grow on longline ropes and moorings at depths in excess of 4 m and the natural host for this species is the "Black Coral" (Order Antipatharia), a gorgonian common in high current, 10- to 20-m deep water.

Summer–autumn spawning peaks have been reported for *Pt. penguin* in Tonga (Malimali 1995, Tanaka 1997) with heavy settlement between March and May 1995, and further research at Orpheus Island has provided similar results. The clearly defined spawning season of *Pt. penguin* at Orpheus Island contrast with *Pteria sterna* in Mexico which spawns throughout the year (Ramirez et al. 1992); however, the depth trends vary with season. *Pt. sterna* settled at highest densities at relatively shallow depths (4 to 7 m) in winter (Caceres–Martinez et al. 1992) and deeper (11–15 m) in summer (Caceres–Martinez et al. 1992, Monteforte et al. 1995).

E. papillionacea was the most abundant of all the bivalves collected and may present a considerable negative factor for collection of commercial species of pearl oysters. *E. papillionacea* is very small (<16 mm) with a life span of three to five months (A. Beer, unpublished data 1999). It is not a commercial species and is likely to have considerable economic importance as a significant fouling species on spat collectors.

In summary, 20 species from three genera (*Pinctada, Pteria,* and *Electroma*) recruited onto spat collectors during this study: eight species of *Pinctada* (*P. margaritifera, P. fucata, P. albina, P. maculata, P. maxima, P. chemnitzii,* and *P. nigra*); eight species of *Pteria* (*Pt. penguin, Pt. cypesyllus , Pt. falcata, Pt. coturnix, Pt. lata, Pt. bernhardi, Pt. Cooki, and Pt. chinensis*); and four species of *Electroma* (*E. alacorvi, E. malleus, E. zebra,* and *E. papillionacea*). This study also demonstrated that spat collection in Pioneer Bay, Orpheus Island, has the potential to supply significant numbers (4,000 *Pinctada sp.* and 1,200 *Pteria sp.*) of juvenile pearl oysters for pearl oyster culture operations.

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IN VITRO KILLING OF *PERKINSUS MARINUS* BY HEMOCYTES OF OYSTERS *CRASSOSTREA VIRGINICA*

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ABSTRACT A colorimetric mircobicidal assay was adapted, optimized, and applied in experiments to characterize the *in vitro* capacity of eastern oyster (*Crassostrea virginica*) hemocytes to kill cultured isolates of *Perkinsus marinus*, a protozoan parasite causing a highly destructive disease of oysters in U.S. Atlantic and Gulf of Mexico coastal waters. *In vitro* challenges showed that hemocytes from two geographically distinct oyster stocks (Florida and Rhode Island) were able to reduce viable *P. marinus* cells by 25% to 90%, depending on the *P. marinus* isolate. Variability in killing was most likely due to differences in susceptibility among the 7 cultured isolates of *P. marinus*, which ranged in origin from Long Island Sound (Connecticut) to Laguna Madre (Texas). Hemocytes from oysters collected monthly from Escambia Bay, Florida, exhibited a killing capacity that ranged from 21% to 90% (average 57%) throughout a year-long period, with highest killing measured from October to December. Application of this technique demonstrated the *in vitro* capacity of hemocytes to kill cultured *P. marinus*, but does not necessarily reflect their ability under natural conditions where the disease is widespread.

KEY WORDS: *Perkinsus marinus*, eastern oysters, *Crassostrea virginica*, bivalve defenses, cellular defense, invertebrate immunology, bactericidal activity

INTRODUCTION

For nearly 50 years, eastern oyster (*Crassostrea virginica*) populations along the U.S. Atlantic coast and Gulf of Mexico have been infected by a highly virulent and transmissible pathogen *Perkinsus marinus* (Mackin et al. 1950, Andrews and Hewatt 1957, Quick and Mackin 1971, Andrews 1988, Burreson et al. 1994). Recently the disease has spread into the northeastern U.S. (Ford 1996), a region previously considered too cold to support *P. marinus*. Prevalence and intensity of *P. marinus* throughout its geographical range are highly influenced by temperature and salinity (see reviews by Andrews 1988, 1996, Burreson and Ragone-Calvo 1996, Soniat 1996). In the Gulf of Mexico, prevalences approach 100% year-round in areas with salinities above 6 ppt (Soniat 1996). In the Chesapeake Bay, prevalences approach 100% during dry, summer periods, but decline during the cool, wet winters (Andrews 1988, Burreson and Ragone-Calvo 1996). Once established, infections will retard oyster growth (Andrews 1961, Paynter and Burreson 1991) and may ultimately be lethal. The high prevalence combined with high mortality have had a devastating effect on eastern oyster populations, populations that are not only an economic asset, but serve critical ecological roles as benthic substrate, secondary producers, and a link between pelagic and benthic food webs (Kennedy 1996).

Among marine invertebrates, oyster antimicrobial defenses have been relatively well studied (see Fisher 1988). Hemocytes are considered the primary line of oyster defense, largely because of their ability to phagocytose and destroy invading microorganisms, but also becasue they function in inflammatory response, wound repair, and encapsulation (Cheng 1979, Fisher 1986). However, the effectiveness of these putative defense mechanisms against *P. marinus* is questionable since oysters do not appear to suppress progression or transmission of the disease (Anderson 1996). Some investigators have found, using electron microscopy, that hemocytes appear to degrade intracellular *P. marinus* (La Peyre 1993, Bushek et al. 1994), while others report that *P. marinus* cells are able to survive and multiply within eastern oyster hemocytes (Perkins 1996). Studies to elucidate these hemocyte-*P. marinus* interactions were previously limited by the difficulty of isolating the pathogen from host tissues. Development of continuous culture techniques for *P. marinus* (Gauthier and Vasta 1993, Kleinschuster and Swink 1993, La Peyre et al. 1993, La Peyre 1996) now enables a closer examination of this interaction and may lead to a better understanding of the inability of oyster defenses to prevent this disease.

A colorimetric technique to estimate killing of bacteria by oyster hemocytes was recently introduced (Volety et al. 1999a). Bacteria and hemocytes were incubated separately and together in wells of a microtiter plate during a challenge period, followed by a grow-out period where surviving bacteria were allowed to multiply to numbers that could be detected after reaction with a tetrazolium salt [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2-*H*-tetrazolium], or MTS. The MTS was added with phenylmethasulfazone (PMS) to the microtiter plate wells where they were enzymatically reduced by living cells, in proportion to their numer, to yield a colored, water-soluble formazan. The absorbance of this reduction product was used to estimate numbers of live bacteria, which was then converted to a

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killing index, i.e. the percent of bacteria killed or rendered nonreproductive by the hemocytes. Much like bacterial plate counts, the assay does not distinguish bacteria survival from their ability to multiply. Nonetheless, a bactericidal/microbicidal terminology has been adopted as a convenient generalization. Successful application of the bacterial killing assay (Genthner et al. 1999) prompted further research to adapt the technique to evaluate *P. marinus* killing by oyster hemocytes.

In this study the MTS/PMS colorimetric technique is adapted, optimized, and subsequently applied in two experiments intended to characterize the variability of oyster hemocyte capacity to kill *P. marinus*. Previous investigators (Dungan and Hamilton 1995) have documented that living *P. marinus* cells can reduce MTS/PMS tetazolium salt to a formazan product that is proportional to the living biovolume (and approximate cell number). The first experiment compares hemocytes from two geographically different oyster stocks in their ability to kill 7 different strains of cultured *P. marinus*, and the second experiment documents monthly changes in killing ability of hemocytes from oysters at a single field location during a year-long period.

METHODS AND MATERIALS

Development and Optimization of the Microbicidal Assay for P. marinus

Oysters were collected from Bayou Texar, an inlet of Escambia Bay, Florida, between August 1998 and July 1999 and transported in coolers to the laboratory of the U.S. Environmental Protection Agency Gulf Ecology Division (GED) to be used for a series of experiments. On days of collection, ambient salinities ranged from 10 to 16 ppt and ambient water temperatures ranged from 10 to 29 °C. At GED, oysters were held in 1,900-L holding tanks equipped with a flow-through raw seawater delivery system for no longer than 1 wk prior to experimental use (for the seasonal studies, experiments were performed on the day of collection). Through the entire holding period, salinity ranged from 15 to 20 ppt and temperatures 10 to 30 °C. Hemocytes in whole hemolymph were collected from the adductor muscle through a notch in the oyster shell using a syringe fitted with a 23-gauge needle. Hemolymph was placed in polypropylene tubes and submerged in an ice bath to reduce hemocyte clumping. For some studies, hemolymph samples were pooled from several (4–10) oysters and for others, hemolymph samples from individual oysters were used.

A single isolate of cultured *P. marinus* was used for all experiments involved in the development and optimization of the assay. This isolate (YR-VA) was originally cultured from infected oysters in the York River, Virginia, in 1992. It was held aseptically for 7 y with successive bimonthly culture transfers using Dulbecco's modified Eagle's medium and F-12 HAM's nutrient mixture (DMEM/F12; Gauthier and Vasta 1993). Parasite cultures for all studies were less than 2 wk old and upon microscopic examination, YR-VA cells appeared healthy with a size distribution of 4 to 40 μm and the majority of cells at 4 to 10 μm. Densities of *P. marinus* in all experiments were determined by microscopic hemocytometer counts.

The killing assay was performed in a manner similar to that described for bacterial killing by oyster hemocytes (Volety et al. 1999a) using formation of colored formazan (reduced from a tetrazolium salt) to distinguish living from dead (or nonproliferating) cells. Briefly, the assay involved incubation of oyster hemocytes and YR-VA, both separately and together for a 3-h challenge period, a grow-out period of surviving YR-VA in *P. marinus* cell culture medium, and a formazan formation period with MTS/PMS solution. Formazan, the reduction product of MTS/PMS, was measured at 490 nm using a microplate reader (Bio-Tek Instruments Model 311-SX). Replicate wells (4–8) were measured and results averaged for calculation of percentage killing, or killing index (KI), as follows:

$$KI\ (\%) = [1 - (A_{H+Pm} - A_H)/(A_{Pm})] \times 100$$

Equation subscripts refer to absorbances measured from wells with hemocytes only (H), *P. marinus* only (Pm), or both (H + Pm). All experiments were performed at 20 ppt salinity, a condition suitable for both *P. marinus* and oyster hemocytes.

To optimize the microbicidal assay for *P. marinus*, some of the same conditions used for bacterial killing (Volety et al. 1999a) were initially adopted. However, the slower growth rate of *P. marinus* necessitated a longer grow-out period (22–24 h) to attain numbers high enough for colorimetric sensitivity. From this initial protocol several different procedural modifications were examined (see below) to determine the influence of these factors on assay results and to characterize the most responsive methodology. As they were identified, the most responsive conditions (higher killing) were assumed for subsequent experiments. In all cases, absorbance was measured at 490 nm. Where appropriate, ANOVA was used to determine the difference in KI resulting from different formazan formation periods, *P. marinus*:hemocyte ratios, volumes of medium, challenge durations, and assay temperatures. As necessary to meet the assumptions of ANOVA, data were transformed either by $\log_{10}$ or arcsin. When significant differences in means were found using ANOVA, Tukey's multiple comparison test was employed to resolve the differences due to treatments. All statistical analyses were performed using SAS (Statistical Analysis Systems, Cary, NC).

Formazan Formation Period

Cells of *P. marinus* (YR-VA) were harvested from culture flasks, counted with a hemocytometer, and diluted with DMEM/F12 cell culture medium (Gauthier and Vasta 1995) to a density of 5×10^6 mL^{-1}. This was then diluted serially to obtain 6 different cell densities (0%, 6.3%, 12.5%, 25%, 50%, and 100%) used to simulate a range of cells surviving a challenge with hemocytes. Aliquots (100 μL) from each density were placed in microtiter wells (N = 8 replicate wells) and held for 22 to 24 h at 28 °C. After this grow-out period, 20 μL of MTS/PMS were added. Absorbance was measured after 1, 2, and 3 h at 28 °C.

Challenge Ratio of YR-VA to Oyster Hemocytes

Three different ratios of *P. marinus* cells to oyster hemocytes were examined to determine effects on assay results. Hemocytes from 10 oysters were pooled and for each challenge ratio, 1×10^5 hemocytes were placed in 8 replicate microtiter wells with appropriate numbers of YR-VA to achieve ratios of 10:1, 5:1, and 2.5:1 *P. marinus* cells per oyster hemocyte. Hemocytes were diluted with sterile-filtered (0.22 μm) sea water at 20 ppt salinity (FSW). Experiments were performed in a final volume of 100 μL FSW. The challenge period was 3 h at 17 °C, followed by a 22-h grow-out and a 3-h formazan formation period, both at 28 °C.

Volume of Sea Water during Challenge Period

The influence of FSW on the assay was examined by comparing the results obtained with four different volumes (25, 50, 100, and 200 μL). Hemocytes from 10 oysters were pooled and for each test volume, 1×10^5 hemocytes were placed in 8 replicate microtiter wells with 1×10^6 YR-VA (challenge ratio = 10:1 *P. marinus* per oyster hemocyte) in FSW. The challenge period was 3 h at 17 °C, followed by a 22-h grow-out and a 3-h formazan formation period, both at 28 °C.

Challenge Duration

The length of time that *P. marinus* were exposed to oyster hemocytes was also examined. Hemocytes from 10 oysters were pooled and for each challenge duration tested, 1×10^5 hemocytes were placed in 8 replicate microtiter wells with 5×10^5 YR-VA (challenge ratio = 5:1 *P. marinus* per oyster hemocyte) and 100 μL FSW. The challenge period was varied from 1 to 5 h at 17 °C, followed by a 22-h grow-out and a 3-h formazan formation period, both at 28 °C.

Assay Temperature

To determine the effect of temperature on hemocyte killing activity, the challenge portion of the assay was performed at five different temperatures (5, 12, 17, 29, and 37 °C). Hemocytes from 10 oysters were pooled and for each challenge temperature, 1×10^5 hemocytes were placed in 8 replicate microtiter wells with 5×10^5 YR-VA (challenge ratio = 5:1 *P. marinus* per oyster hemocyte) and 100 μL FSW. The challenge period was followed by a 22-h grow-out and a 3-h formazan formation period, both at 28 °C.

Comparison of KI with Direct Counts

Hemocytes from each of 10 individual oysters were placed in five wells (10^5 per well) in a 96-well microtiter plate. *P. marinus* (YR-VA; 5×10^5 per well) were added to the hemocytes for a 3-h challenge at 17 °C and to replicate wells in a separate plate that contained no hemocytes. At the end of the challenge period, *P. marinus* culture medium (DMEM/F12) was added for 22 h at 28 °C, enabling the surviving cells to proliferate. After the grow-out period, 4 of the 5 replicate wells received 20 μL MTS/PMS for 3 h at 28 °C prior to measurement of absorbance. The fifth replicate well was aspirated thoroughly and a 50% concentration of trypan blue dye was added to the wells for 5 to 10 min. The contents were aspirated and 2 aliquots placed on a hemocytometer where living *P. marinus* cells were counted at 40×. Dead cells take up trypan blue dye and appear blue, whereas live cells are not stained. The average number of live *P. marinus* from the two counts was subtracted from the live average in the control (no hemocyte) wells and the percentage dead calculated for each oyster. (Because some hemocytes may have engulfed and masked some *P. marinus* from exposure to trypan blue, the percentage dead may be somewhat underestimated.) Killing index, calculated for each oyster from the average of four wells measured colorimetrically, was compared wth the percentage dead (by count). A paired *t* test was used to determine whether significant differences ($P \leq 0.05$) existed between means ($n = 10$) of KI obtained by tetrazolium salt reduction and direct count methods, and Pearson's correlation analysis was used to compare the methods.

Susceptibility of Different Cultured* P. marinus *Isolates to Hemocyte Killing

Seven cultured *P. marinus* isolates were tested against oyster hemocytes (Table 1). These included isolates originally cultured from infected oysters collected from Long Island Sound, Connecticut (LI-CT), Delaware Bay, New Jersey (DB-NJ), Oxford, Maryland (OX-MD), Mobjack Bay, Virginia (MB-VA), York River, Virginia (YR-VA), Barataria Bay, Louisiana (BB-LA), and Laguna Madre, Texas (LM-TX). Cultures of all these isolates were initiated from infected oysters between June 1991 and October 1992. These isolates have been used to investigate mechanisms of infection (Volety and Chu 1995), host-parasite interaction (Bushek and Allen 1996b, Bushek et al. 1997), different races of *P. marinus* (Bushek and Allen 1996a), population genetics (Reece et al. 1997), and potential virulence factors (La Peyre et al. 1996). Since isolation, cultures have been maintained on *P. marinus* culture medium (Gauthier and Vasta 1993, Gauthier and Vasta 1995) with transfers every 2 to 4 wk. Cultures for all experiments were less than 2 wk old and all cells appeared healthy upon microscopic examination.

Two geographic sources of oysters (stocks) were used to compare susceptibility of different *P. marinus* isolates. Oysters were collected from Bayou Texar in Escambia Bay (FL) on May 6 and May 10, 1999 where ambient water temperature was 23 to 26 °C and salinity was 13 to 16 ppt. They were held overnight in tanks with running sea water (25 °C and 21 ppt salinity) prior to assay. Oysters were also collected from Bissel Cove, Rhode Island on June 15, 1999 where ambient temperature was 20 °C and salinity was 30 ppt. Oysters were shipped overnight in a cooler with ice to the GED laboratory where they were acclimated to 20 °C and 20 ppt over 5 d prior to assay. Two trials were performed for each oyster stock. For each trial, 4 to 5 pools of hemocytes drawn from 3 to 5 oysters were challenged with *P. marinus*. The assay was performed simultaneously for all 7 *P. marinus* isolates and all pools of hemocytes from a single oyster stock. The protocol consisted of a 3-h challenge period at 17 °C with a 5:1 ratio of *P. marinus* per oyster hemocyte, followed by a 24-h grow-out period and a 3-h formazan formation period at 28 °C. KI was calculated for each pool of hemocytes and results for each pool in a trial were averaged. Because the two oyster stocks were collected at different times of year, each was considered a separate experiment. Two-way ANOVA was performed separately for each oyster stock to determine differences in KI due to main effects of *P. marinus* isolate and trial number and Tukey's multiple comparison test was applied if significant differences were found.

TABLE 1.

***P. marinus in vitro* isolate cultures.**

Isolate	Geographic Source	ATCC Code
LI-CT	Long Island Sound, CT	50508
DB-NJ	Delaware Bay, NJ	50509
OX-MD	Choptank River, MD	50439
MB-VA	Mobjack Bay, VA	50510
YR-VA	York River, VA	—
BB-LA	Barataria Bay, LA	50511
LM-TX	Laguna Madre, TX	50512

Seasonal Killing Ability of Oyster Hemocytes

Oysters were collected monthly from Bayou Texar (Escambia Bay, Florida) and transported in coolers to GED. Ambient water salinity and temperature measurements were made at each time of collection. Ten oysters were arbitrarily selected and hemolymph drawn from the adductor muscle as described above. Hemocyte densities were determined and the microbicidal assay was performed using hemocytes from each individual oyster and the *P. marinus* isolate YR-VA. Assay protocol was the same as that used for examining the susceptibility of different *P. marinus* isolates. Rectal tissues from the sampled oysters were processed with fluid thioglycollate medium to determine infection intensity of *P. marinus* (Ray 1966), and individuals assigned a value based on the Mackin scale (Craig et al. 1989). Several simple linear regression analyses were used to determine the association of independent variables temperature and salinity with monthly mean hemocyte densities and KI. Pearson's correlation analyses (SAS, Cary, North Carolina) were conducted to evaluate the relation between monthly mean hemocyte densities and KI and *P. marinus* infection intensity and KI.

RESULTS

Development and Optimizatian of the Microbicidal Assay for **P. marinus**

Preliminary results indicated that a 22- to 24-h grow-out period of *P. marinus* cells was needed to allow sufficient proliferation to detect changes in absorbance. Cells of *P. marinus* incubated with MTS/PMS showed an increase in absorbance at higher densities (Fig. 1). Regardless of the period allowed for formazan formation, absorbance was highly correlated with cell density (r = 0.96–0.97). The slope of the relationship increased with increasing formazan formation. Killing indices obtained using challenge ratios of 2.5:1 (KI = 80.0%) and 5:1 (KI = 75.2%) *P. marinus* cells per oyster hemocyte were statistically greater ($P = 0.0001$) than that derived from the 10:1 ratio (KI = 17.0%, Fig. 2). The KI from microtiter wells containing 100 µL FSW (KI = 66.5%) was significantly higher ($P = 0.0001$) than indices obtained from wells with higher and lower volumes (Fig. 3). No significant differences were detected in KI among challenge periods that were 1, 2, 3, or 5 h (Fig. 4), but the 4-h challenge was lower than all other challenge durations ($P = 0.001$). Although there were no statistically significant differences in KI among assay temperatures of 5, 12, 17, and 37 °C, KI at a challenge temperature of 29 °C was significantly lower ($P = 0.0001$) than those obtained at 5, 12, and 17 °C (Fig. 5).

Killing indices for 10 individual oysters, using the formazan technique, averaged 61.4% (±12.1 SD) as compared to an average 57.1% (±15.4 SD) estimated by direct counts made after incubation in DMEM/F12 cell culture medium. The greatest discrepancy between methods for an individual was 27%, yet discrepancies of 1% or less were found for 3 of the 10 oysters. There was no statistical difference between the average *P. marinus* killing for any of the 10 individuals as measured by the two techniques ($P = 0.29$) and the results were positively correlated ($r = 0.628$, $P < 0.05$).

Susceptibility of Different **P. marinus** *Isolates to Hemocyte Killing*

The Mobjack Bay *P. marinus* isolate (MB-VA) was consistently the most susceptible to oyster hemocyte killing, with KI for individual oysters ranging from 73 to 91% and isolates BB-LA, DB-NJ, and LI-CT (individual KI = 20–46%) were among the least susceptible, regardless of oyster origin (Fig. 6). Average KI for both oyster stocks against all cultured isolates was 48%. There

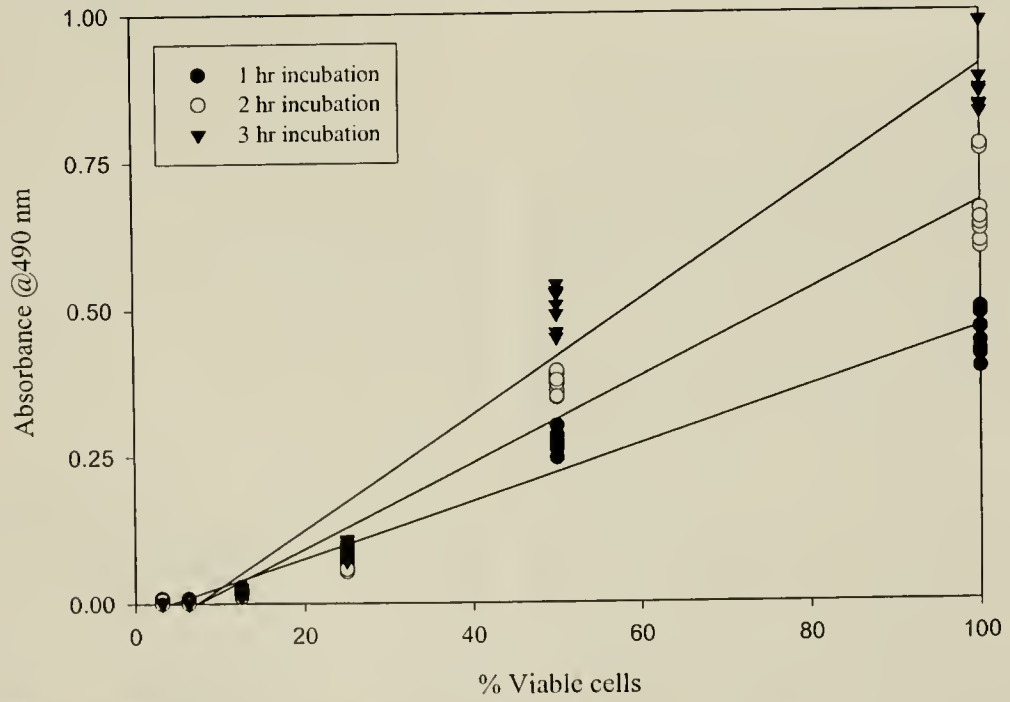

Figure 1. Absorbance readings (490 nm) of microtiter wells containing *P. marinus* (YR-VA) trophozoites at densities of 0, 6.3, 12.5, 25, 50, and 100% of a 5×10^5 cell inoculum after a 22-h grow-out period followed by a 1-h (r = 0.97), 2-h (r = 0.96), and 3-h (r = 0.97) incubation with MTS/PMS reagent.

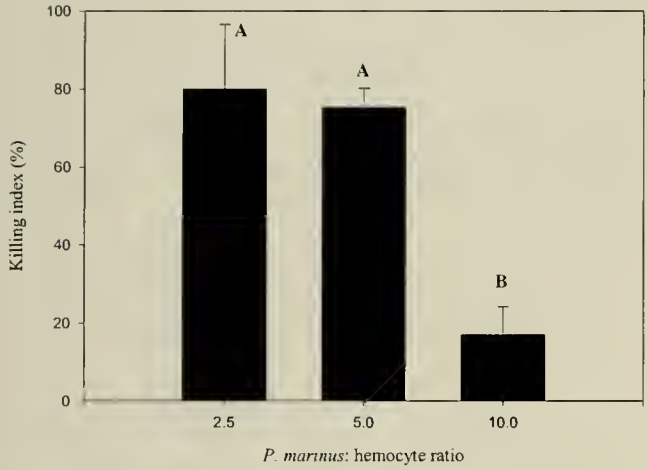

Figure 2. Mean KI (±SD) for *in vitro* challenge ratios of 2.5:1, 5:1, and 10:1 *P. marinus* trophozoites (YR-VA) per oyster hemocyte. Different letters designate statistically different means (Tukey's multiple comparison, $P < 0.05$).

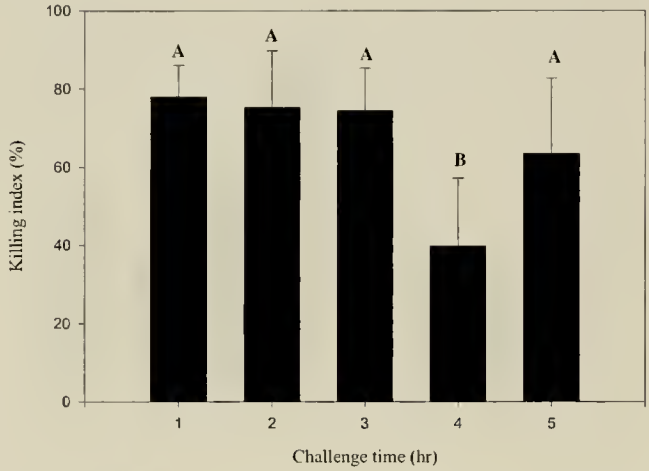

Figure 4. Mean KI (±SD) for *in vitro* challenges between oyster hemocytes and *P. marinus* (YR-VA) ranging from 1 to 5 h for the challenge period. Different letters designate statistically different means (Tukey's multiple comparison, $P < 0.05$).

was no significant difference due to trial in the Rhode Island oyster challenges (ANOVA, $P = 0.08$) and Tukey's multiple comparison test found KI of isolate MB-VA to be significantly higher than all other isolates. A significant difference due to trial was detected for Florida oyster challenges (ANOVA, $P = 0.001$).

Seasonal Killing Ability of Oyster Hemocytes

The capacity of oyster hemocytes to kill YR-VA varied over the sampling period, with monthly average KI ranging from 21 to 90% (Fig. 7) and an average 57% across all months of the year-long monitoring period. Killing index was lowest in July and August (average KI = 21–41%) and highest in October through December (average KI = 75–90%). Correlation analysis found KI to vary inversely with salinity and temperature measured at the collection site at the time of collection. Simple linear regression analysis of KI with salinity was significant ($r = -0.75$, $P = 0.006$), whereas that with temperature was not ($r = -0.50$, $P = 0.11$). Hemocyte density was not clearly associated with KI ($r = -0.26$, $P = 0.42$) nor temperature ($r = 0.51$, $P = 0.09$), but was weakly associated with salinity ($r = 0.59$, $P = 0.04$). Prevalence of *P. marinus* was 100% throughout the study period and infection intensity ranged from 2.5 to 4.1 (scale of 0–5). Neither individual oyster KI nor monthly average KI was significantly associated (Pearson's) with infection intensity.

DISCUSSION

An *in vitro* microbicidal assay, previously developed to estimate susceptibility of bacteria to killing by eastern oyster hemocytes (Volety et al. 1999a), was adapted and used to characterize the capacity of hemocytes to kill *P. marinus* cells. *In vitro* challenges showed that hemocytes from two geographically distinct oyster stocks were able to eliminate 25 to 90% of *P. marinus* cells grown from cultures isolated at 7 different locations along the Gulf of Mexico and U.S. Atlantic coast (Fig. 6). This finding, coupled with the year-round average of 57% KI of one isolate by hemocytes from Florida oysters (Fig. 7), implies that hemocytes may have some capacity to combat this destructive disease agent. Such an implication is supported by at least a few reported obser-

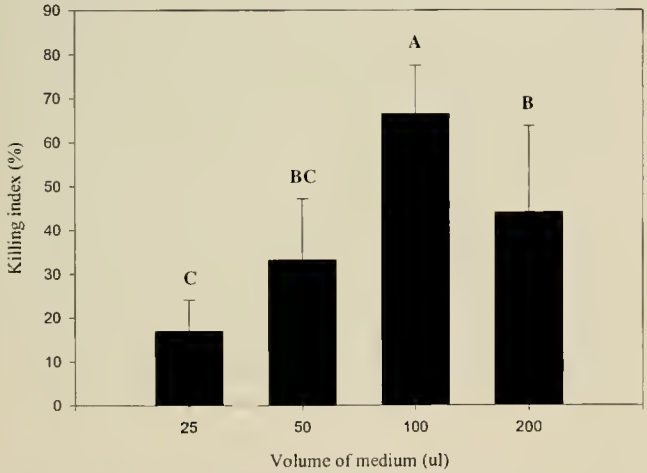

Figure 3. Mean KI (±SD) for *in vitro* challenges between oyster hemocytes and *P. marinus* (YR-VA) using 25 to 200 µL sea water in microtiter wells. Different letters designate statistically different means (Tukey's multiple comparison, $P < 0.05$).

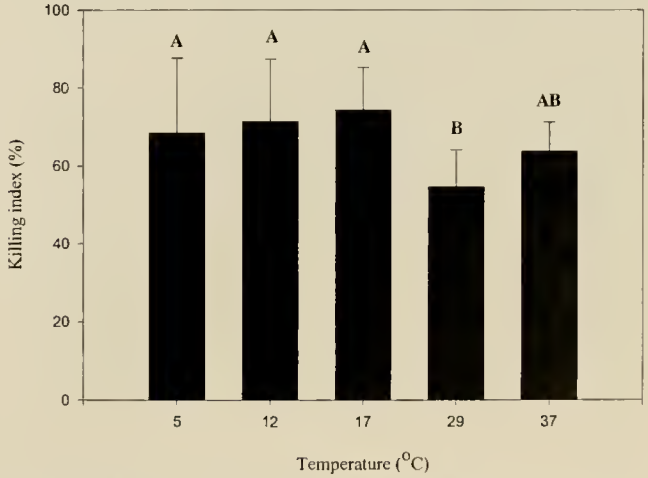

Figure 5. Mean KI (±SD) for *in vitro* challenges between oyster hemocytes and *P. marinus* (YR-VA) ranging from 5 to 37 °C for the challenge period. Different letters designate statistically different means (Tukey's multiple comparison, $P < 0.05$).

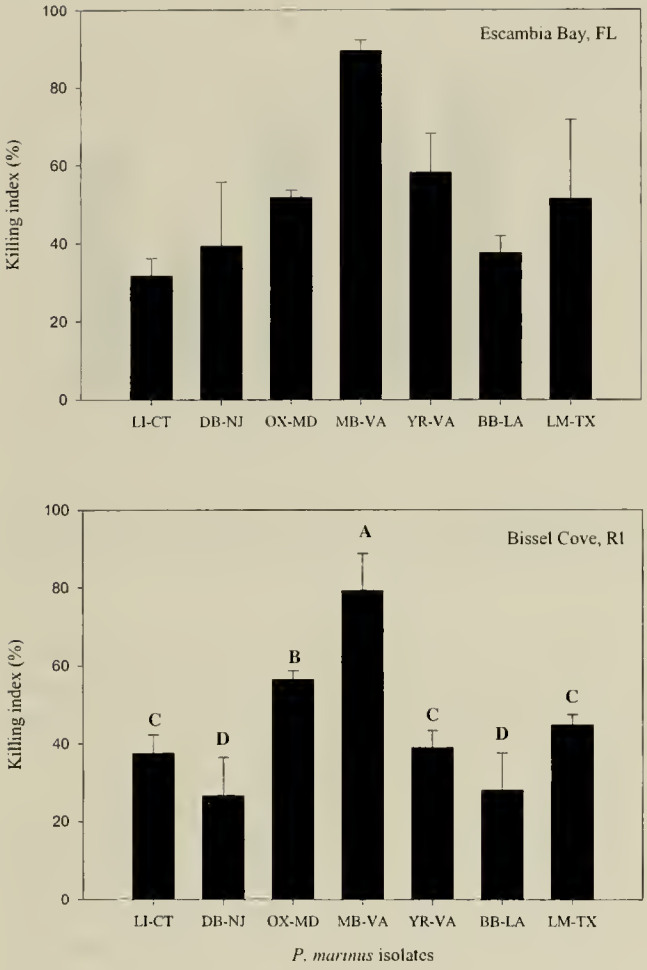

Figure 6. Mean KI (±SD) for 7 different *P. marinus* isolates challenged *in vitro* with hemocytes from oysters collected in Escambia Bay, Florida (top) and Bissel Cove, Rhode Island (bottom). Bars represent the combioed average of two trials and for Rhode Island oysters (bottom), there was no difference between trials (ANOVA). Different letters designate statistically different means (Tukey's multiple comparison, $P < 0.05$).

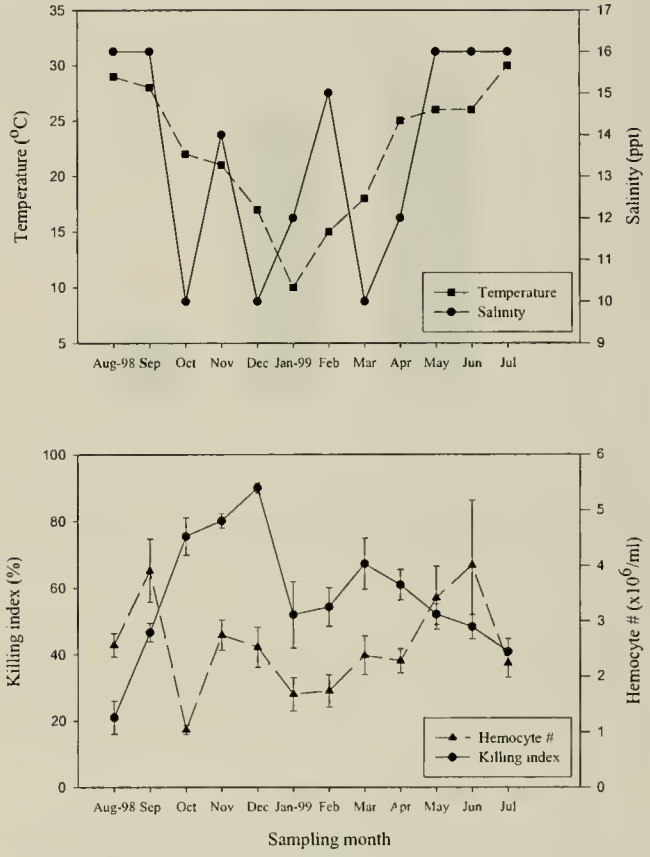

Figure 7. Monthly monitoring of oysters in Escambia Bay, Florida during a 1-y period in 1998 and 1999: (top) ambient water temperature and salinity measured at the time of oyster collectioo; (bottom) mean hemocyte density (±SD) and KI (±SD) calculated from independent tests on 10 individual oysters.

vations of phagocytic destruction of *P. marinus* in field-collected oysters (La Peyre 1993, Bushek et al. 1994).

However, further investigation is required to determine whether oyster hemocytes can provide a meaningful defense against *P. marinus* in nature. One of the most crucial questions related to evidence provided by this study is whether cultured *P. marinus* isolates accurately retain the vigor of naturally occurring *P. marinus*. Through repeated transfers and lack of contact with oyster tissues, cultured parasites may have lost the ability to protect themselves against host defenses, typically accomplished through masking (Mauel 1984, Hall and Joiner 1991) or repellent chemical secretions (Le Gall et al. 1991, Yoshino et al. 1993, Volety and Chu 1995). Perhaps as a consequence, several investigators have reported low virulence of cultured isolates (La Peyre et al. 1993, Gauthier and Vasta 1993, Volety and Chu 1994, Chintala et al. 1995).

Other factors must also be reconciled between natural challenges and the *in vitro* assay conditions presented here. Unlike natural infections where several different sizes and stages of *P. marinus* exist in oysters (Perkins 1996), the cultured cells were relatively homogenous (4–10 μm diameter) and generally lacked hypnospores and tomonts because of their growth on nutrient-rich medium. The *in vitro* chemical environment was unlike natural conditions because the challenge medium did not include hemolymph. Finally, killing in natural conditions may be influenced by a variety of external conditions such as temperature, salinity, or the nutritional and gametogenic status of the oyster. Ultimately, *P. marinus* is known to persist, and even flourish in oysters from Mexico to Maine (Burreson et al. 1994, Ford 1996). So, whatever killing capacity oyster hemocytes may have, their ability under natural conditions is insufficient to offset *P. marinus* rates of multiplication (Powell et al. 1996) and to eliminate disease from the oyster population.

An important technical difference exists between this method for *P. marinus* and the bactericidal assay (Volety et al. 1999a). In the *P. marinus* method, the post-challenge cell culture medium does not affect hemocyte survival, so the 22- to 24-h grow-out period for *P. marinus* multiplication may simultaneously allow continued hemocyte killing. This probably created the relatively consistent KI (except for the 4-h duration) found when the challenge period was varied from 1 to 5 h (Fig. 4). An additional 22-h period for killing may have overshadowed differences that occurred between 1 and 5 h. An extended killing period may also explain the lack of significant differences in KI at assay temperatures of 5 to 29 °C (Fig. 5). After the 3-h challenge period in these experiments, a grow-out period proceeded for an additional 22-h at 28 °C, conditions sufficiently similar to 29 °C to mask any effects of temperature differences applied for only 3 h. Because of the

TABLE 2.

Recommended protocol to estimate killing of *P. marinus* by oyster hemocytes.

1. One week prior to assay, start a *P. marinus* culture ($1–5 \times 10^6$ cells/mL) in 1:2 DMEM/F-12 nutrient mixture (Gauthier and Vasta 1995) from a culture no more than 1-mo-old. Maintain cultures at 28 °C.
2. Collect hemolyph from the adductor muscles of oysters using a 1 to 3 mL syringe fitted with a 23-gauge needle. Place the hemolymph into polypropylene tubes in an ice bath to reduce hemocyte aggregation. Estimate hemocyte numbers using a hemocytometer and calculate the volume of hemolymph needed to yield 1×10^5 hemocytes for each microtiter plate well.
3. Before adding oyster hemolymph, add 100 µL FSW (filter-sterilized, 20 ppt) into the wells of a microtiter plate. To the H and H + P wells, add the volume of hemolymph needed for 1×10^5 hemocytes. Centrifuge the microtiter plate at 160*g* for 10 min to ensure hemocyte adhesion in a monolayer.
4. Gently remove hemolymph plasma/FSW from the microtiter plate wells using a multichannel pipet (leave the hemocyte monolayer moist). Quickly add 100 µL FSW to control blank wells and H only wells. Add 100 µL FSW containing 5×10^5 *P. marinus* to H + P and P only wells.
5. Centrifuge microtiter plate at 160*g* for 10 min to ensure contact of *P. marinus* with hemocytes.
6. Maintain microtiter plates in a humid chamber at 17 °C for 3-h challenge period.
7. Add 100 µL *P. marinus* cell culture medium (DMEM/F-12) to all wells. Maintain the plate at 28 °C for a 22 to 24 h *P. marinus* grow-out period.
8. Add 20 µL MTS/PMS reagent to each well and incubate for 3 h at 28 °C to allow color development.
9. Measure absorbances of the wells at 490 nm using a microplate reader set to subtract the absorbance of blank wells from sample wells.
10. Calculate KI as follows:

$$KI\ (\%) = [1 - (A_{H+Pm} - A_H)/(A_{Pm})] \times 100$$

likelihood of simultaneous killing during the parasite grow-out period, a separate challenge period may be duplicative. Alternatively, future iterations of the technique could concentrate on a means to kill or disable the hemocytes at the end of the challenge period (without damaging the parasites).

Hemocytes withdrawn from two geographically distinct stocks of oysters (Florida and Rhode Island) exhibited similarities in their ability to kill different isolates of *P. marinus* (Fig. 6). Hemocytes from both oyster stocks were more able to kill MB-VA and less able to kill BB-LA, DB-NJ, and LI-CT. Because of this similarity, variations in the results appeared to stem from differences in susceptibility of the isolates rather than differences in the killing ability of hemocytes from the two oyster stocks. There was no evidence of geographic differences in killing ability and no evidence to account for observations (Bushek 1994, Bushek and Allen 1996b) that Atlantic coast isolates of *P. marinus* (DB-NJ and MB-VA) generate higher infection intensities than Gulf coast isolates (BB-LA and LM-TX). It is possible that different culture histories influenced the condition and vigor of the various cell lines, but susceptibility may also be influenced by genetic variability among races of *P. marinus* (Bushek and Allen 1996a, Reece et al. 1997).

Florida oyster hemocytes were capable of killing *P. marinus* YR-VA throughout the year-long monitoring period (Fig. 7). KI was lowest during July and August, but climbed dramatically through December. If this seasonal pattern is typical, these data imply a mechanistic link for reduced killing capacity with high temperature, high salinity, and post-reproductive condition (Andrews 1988, Burreson and Ragone-Calvo 1996, Soniat 1996). Association of killing capacity with intensity of natural *P. marinus* infections was not found, yet interpretation of such data should be viewed with caution since it is likely that only oysters with relatively low infections survive during the warm summer months (Fisher et al. 1992).

The greater capacity of Florida oyster hemocytes to kill *P. marinus* during the winter (Fig. 7) contrasts with their low winter bactericidal capacity for *Vibrio parahaemolyticus* and *Lysteria monocytogenes* (Genthner et al. 1999, Volety et al. 1999b). In addition, the positive correlation between oyster hemocyte density with bactericidal activity (Volety et al. 1999b) was not observed here. These discrepanices may signal different *in vitro* hemocyte killing mechanisms for bacteria and *P. marinus*.

The results presented here demonstrate unequivocally that oyster hemocytes *in vitro* have the capacity to kill *P. marinus*. Moreover, the technique that was developed provides an efficient and defendable tool for examining interactions of oyster defenses with *P. marinus* and other microorganisms. The protocol recommended from optimization studies (Table 2) is relatively simple, inexpensive, and can be easily performed with numerous repetitions. Similar responses were obtained with hemocytes from two distinct oyster stocks and there was good correspondence between the colorimetric technique and direct microscopic counts. We anticipate that this technique will be used in the future to investigate potential differences between hemocyte killing capacity and killing ability in natural conditions. It may also play a key role in elucidating other host-parasite interactions, such as the role of external environmental factors, mechanisms of action, and differences in defense responses to different microbial invaders.

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A METHOD FOR PRESERVING OYSTER TISSUE SAMPLES FOR FLOW CYTOMETRY

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ABSTRACT Flow cytometry (FCM) is a powerful method for ploidy determination which has become important because of the increasing use of triploids in aquaculture. Tissue samples for FCM can be biopsied and kept fresh or frozen in a staining solution containing dimethyl sulfoxide (DMSO). Samples can be stored in the stain/DMSO at −80 °C indefinitely, or shipped on dry ice to a flow cytometry lab. But ultracold freezers and overnight shipping are not always available, for example, at rural labs and hatcheries. We investigated several methods of preserving FCM samples that do not involve freezing. Three different tissues, gill, mantle, and hemolymph from diploid and triploid Pacific oysters, *Crassostrea gigas* Thunberg, were preserved by different methods, including pre-treatments and different fixatives. Gill was the best tissue for FCM analysis, and ethanol (75%) was the preferred fixative. Hypotonic treatments before fixation promoted nucleus-dissociation needed for FCM. The recommended protocol for preserving gill tissue is to dissect or biopsy a piece gill tissue (~0.5 cm^2), treat with 0.075 M KCl for 10 min and fix in 75% ethanol that is changed once. Before FCM, the fixed tissue is washed once using a phosphate buffered saline (pH = 6.8) and transferred to a staining buffer containing 10% DMSO. The stained sample is frozen and thawed, votexed, aspiarted, and filtered before analysis. This method can also be used for preserving D-stage larvae and gill tissue samples of other bivalve species.

KEY WORDS: Triploidy, polyploidy, DNA content, flow cytometry, fixation, oyster, mollusc

INTRODUCTION

Chromosome set manipulation is an important field in shellfish genetics and breeding. Triploid molluscs are important for aquaculture because of their sterility, high meat yield, and quality (Chew 1994). Tetraploids are valuable because they can produce 100% triploids by mating with normal diploids (Guo et al. 1996). Polyploid induction has been studied in over 20 molluscan species so far (Beaumont and Fairbrother 1991, Guo et al. 1999). During chromosome set manipulation, ploidy determination is a critical and necessary procedure. Methods for ploidy determination include chromosome counting, comparing nuclear size (Utting and Child 1994), electrophoretic assay (Allen et al. 1982), fluorescence staining (Komaru et al. 1988, Uchimura et al. 1989), and flow cytometry (FCM) (Allen 1983). FCM is by far the most powerful method for ploidy determination because of its speed, simplicity, and accuracy. It measures DNA content by recording the fluorescence intensity of nuclei stained with a DNA-specific dye, such as 4,6-diamidine-2-phenylindole (DAPI) or propidium iodide (PI). Ploidy can be determined by analyzing thousands of cells in a few minutes.

Normally, samples for FCM analysis need to be prepared fresh or kept frozen in a staining buffer containing dimethyl sulfoxide (DMSO). While taking samples is relatively straight forward in shellfish, storing them until they reach a flow cytometry laboratory is often difficult. This is especially true for remote laboratories and hatcheries. Shipping samples from remote places often results in severe degradation because tissues can not be kept cold during transport. Individual cells such as hemolymph or isolated nuclei can be fixed for FCM without problem. Solid tissue samples, when fixed, are problematic for FCM. There are several existing methods for isolating nuclei for FCM from fixed tissues, mostly variants of the Hedley method (Hedley et al. 1983, Hedley 1994). These protocols are designed for clinical applications and involve laborous rehydration, enzyme treatments, and washes, and are not practical for rapid ploidy determination of a large number of samples.

The purpose of this study was to find a simple and effective method to preserve tissue samples for FCM analysis without freezing and laborous post-fixation treatment, allowing tissue samples to be stored, transported, and analyzed easily. Three separate experiments were conducted using diploid and triploid Pacific oysters, *Crassostrea gigas* Thunberg, to test different tissues, pretreatments, and methods of fixation. Here we report a simple protocol for tissue preservation and nucleus isolation, which is effective for rapid FCM analysis of ploidy in molluscs.

MATERIALS AND METHODS

Pacific oysters used in this study were 2-y-old diploids and triploids produced by crossing diploids and tetraploids (Guo et al. 1996). FCM analysis was conducted on a Partec Ploidy Analyzer using a staining solution consisting of 10 mg/L DAPI dissolved in a nucleus isolation buffer (10 mM Tris, 146 mM NaCl, adjust pH to 7.4, 2 mM $CaCl_2$, 22 mM $MgCl_2$, 0.1% Triton X-100, and 0.005% bovine serum) with 10% DMSO (DAPI/DMSO, modified from Guo et al. 1993). The following method of sample preparation, which is routinely used in our laboratory, was used as the normal control method in this study. Briefly, the tissue sample (~0.5 cm^2) is placed directly into 1 mL of DAPI/DMSO in a 1.5-mL centrifuge tube and frozen at −80 °C until use. Before FCM, the frozen sample is thawed at room temperature, vortexed,

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aspirated 5 times with a 1-cc syringe (25-gauge needle), and filtered through a 25-μm screen. We conducted three experiments to evaluate different aspects of tissue preservation and preparation.

Experiment I: Direct Fixation versus Pre-Treatment

Hemolymph, gill, and mantle tissues were dissected from 15 diploid and 15 triploid oysters. Hemolymph (0.2–0.3 mL) was drawn from the adductor muscle using a 25-gauge needle, split into 2 aliquots, and kept on ice. Gill and mantle tissues were cut into three pieces (~0.5 cm^2) and kept on ice. One piece/aliquot was prepared by the normal method (frozen) and used as a control. The others were prepared for FCM by the following methods.

Method 1: Direct Fixation

Hemolymph (0.1–0.15 mL), gill, and mantle tissues (~0.5 cm^2) were each immersed in 1 mL of Carnoy's fixative (3:1, absolute methanol:acetic acid) in 1.5-mL tubes. Gill and mantle were further cut into 3 pieces to facilitate complete fixation. Fixative was changed once and then samples were stored at 4 °C for 1 wk.

Method 2: Hypotonic Pre-Treatment

Gill and mantle tissues (~0.5 cm^2) were treated with a hypotonic solution (0.075 M KCl) for 20 min and then cut into 3 pieces in Carnoy's fixative that was changed once. Samples were stored at 4 °C for 1 wk.

Before FCM, the fixed samples were washed once with phosphate buffered saline (PBS, 0.025 M KH_2PO_4, pH = 6.8), transferred to 1 mL of DAPI/DMSO, votexed, aspirated five times, and then filtered through a 25-μm nytex screen. Wash and fixative change were made by pelleting the cell/tissue suspension with centrifugation (1 min at 2,000*g*) and replacing the supernatant.

Experiment II: Pre-Treatments

Gill tissue was dissected from 5 diploid Pacific oysters, and cut into ~0.5 cm^2 pieces. Each piece was treated in the following way before fixation in Carnoy's: (1) hypotonic treatment with deionized water for 5, 10, 15, 20, 25, and 30 min; (2) hypotonic treatment with 0.075 M KCl: treatment times were 10, 15, 20, 25, and 30 min; (3) treatment with detergent, 0.5% Triton X-100 dissolved in 0.85% NaCl, for 10, 15, 20, 25, and 30 min; (4) no treatment, tissue was directly fixed in Carnoy's; and (5) tissue was frozen in DAPI/DMSO (the control method). After pre-treatments, each gill sample was cut into 3 to 5 pieces in Carnoy's fixative that was changed once. These samples were stored at 4 °C for 1 wk. Before FCM, tissue samples were washed once with PBS (pH = 6.8), vortexed in 1 mL of DAPI/DMSO for 10 to 15 sec, aspirated five times with a 1-cc syringe (25-gauge needle), and then filtered through a 25-μm nytex screen.

Experiment III: Fixatives

In this experiment we tested three different fixatives: Carnoy's, 75% ethanol, and 10% formalin. Gill tissue from 3 diploid Pacific oysters were dissected and cut into equal pieces (~0.5 cm^2), each piece was treated with 0.075 M KCl for 5, 10, 15, or 20 min and then cut into 3 pieces in 1 of the 3 fixatives. After changing fixatives once, samples were stored at 4 °C for 1 wk.

For FCM, samples were washed once with PBS (pH = 6.8), frozen in 1 mL of DAPI/DMSO at −80 °C for 1 h, thawed at room temperature, vortexed for 15 to 20 sec, aspirated 5 times with a 25-gauge needle, and filtered through a 25-μm nytex screen.

Data Collection

FCM records, in rapid succession, the fluorescence intensity of nuclei, which are suspended in the DAPI/DMSO staining solution. The FCM histogram so obtained describes the distribution of fluorescence signals from nuclei by a frequency distribution histogram. The peak position of the histogram as measured by channel numbers on the horizontal axis reflects the relative DNA content/nucleus, and the number of nuclei recorded is shown on the vertical axis (counts). The term peak position (in channel numbers) is used to describe the position of the histogram along the x-axis in this report.

For this study the flow cytometer was set to obtain at least 10,000 counts at a rate of 400 to 500 cells/nuclei per second. FCM histograms were analyzed using the curve-fitting program, Modfit™, to estimate peak position and coefficient of variation (CV) of frequency distribution histograms. The peak position to a control peak of known ploidy level was used to determine ploidy level of a given sample. CV was used to evaluate the quality of the distribution peak that is affected by sample preparation. A large CV is an indication of poor sample quality caused by preparation. Difference in peak position and CV was compared by two-sampled *t* test or ANOVA using the statistics package, SYSTAT 6.0 (Wilkinson 1996).

RESULTS

Experiment I: Pre-Treatment versus Direct Fixation

The peak position and CV of hemolymph, gill, and mantle tissue from diploids and triploids prepared by freezing (the control method) and fixation in Carnoy's without (Method 1) or with (Method 2) the hypotonic pre-treatment are presented in Table 1.

For hemolymph, a 0.2-mL sample was enough for FCM and histograms were of good quality. Peak position of directly fixed hemolymph, however, was significantly ($P < 0.001$) higher than that of frozen samples in diploids and triploids (two-sampled *t* test). With the control method the ratio of peak position of triploid to diploid was 1.54. With direct fixation, the 3n/2n ratio was 1.61, primarily because of a shift of the triploid peak position to the right. Hypotonic pre-treatment was not used for hemolymph cells considering that they are single cells.

Gill tissues from diploid and triploid oysters were prepared by freezing and fixation with or without hypotonic pre-treatment. Direct fixation did not change peak position of diploids and triploids compared with the control method (Table 1). Hypotonic pre-treatment before fixation increased the peak position of diploids ($P = 0.024$), but not triploids. Direct fixation with Carnoy's affected the quality of the histogram and resulted in higher CVs than the control method. Comparatively, pre-treatment with the hypotonic solution did not affect the quality or CV of the histograms. An important difference between fixation with and without pre-treatment was the number of nuclei produced. Samples pre-treated with the hypotonic treatment produced higher concentrations of nuclei than the directly fixed samples. Sometimes it was difficult to obtain enough nuclei (10,000) for FCM from directly fixed samples.

For mantle tissue, the peak position of directly fixed samples did not differ from that of frozen samples for diploids and triploids. With the hypotonic pre-treatment, peak positions were the same as with the control method for triploids, but higher for diploids ($P = 0.002$). Mantle tissue, when directly fixed, resulted in broad his-

TABLE 1.

Peak position and CV (mean ± SD) of FCM histograms when different tissues of diploid and triploid Pacific oysters were prepared by freezing (control), direct fixation in Carnoy's, and a hypotonic pre-treatment before fixation (n = 15).

Peak		Diploid			Triploid		
		Hemolymph	Gill	Mantle	Hemolymph	Gill	Mantle
Position	Freeze control	50.2 ± 2.6	49.6 ± 1.8	48.6 ± 4.0	77.4 ± 2.6	73.1 ± 3.9	72.9 ± 3.2
	Direct fixation	55.9 ± 3.4	48.6 ± 2.8	50.0 ± 4.2	90.1 ± 3.6	73.1 ± 7.4	75.9 ± 5.0
	P value*	<0.001	0.624	0.296	<0.001	1.000	0.142
	Pre-treatment	—	52.4 ± 3.8	52.0 ± 2.8	—	76.4 ± 4.8	74.6 ± 4.3
	P value*	—	0.024	0.002	—	0.248	0.513
CV	Freeze control	12.0 ± 2.0	11.2 ± 1.6	11.7 ± 1.9	12.4 ± 1.4	10.5 ± 1.4	12.2 ± 1.7
	Direct fixation	10.3 ± 2.6	15.7 ± 2.7	15.8 ± 5.0	10.7 ± 2.0	13.9 ± 2.1	14.8 ± 2.5
	P value*	0.058	<0.001	0.004	0.014	<0.001	0.002
	Pre-treatment	—	9.8 ± 1.7	12.5 ± 2.8	—	11.0 ± 1.4	12.7 ± 1.6
	P value*	—	0.136	0.810	—	0.729	0.765

*P values are from two sample t tests between treatment groups and the control.

tograms. CVs from direct fixation were significantly higher than that from the control method for both diploids and triploids (Table 1). CVs from pre-treated mantles were the same as that from the control method. When fixed, mantle tissue rarely produced adequate numbers of nuclei for FCM, with or without the pre-treatment. All trials with mantle tissue were subsequently discontinued.

Experiment II: Different Pre-Treatments

Because the pre-treatment in Experiment I affected peak position in two cases, different pre-treatments were further tested and compared with direct fixation. Peak position and CV of gill tissues pre-treated with different treatments and for different durations before fixation are presented in Table 2. Varying the time of hypotonic treatment did not change the peak position or CV in any of the three pre-treatments, as suggested by ANOVA. The mean peak position and CV (across pre-treatment durations) of each pre-treatment were compared to that from the control method. Only the Triton X-100 treatment differed from the control method and resulted in lower peak positions and higher CVs than the control. Pre-treatment with KCl or H_2O did not affect peak position and quality.

Sufficient numbers of nuclei were obtained from all pre-treatments. Again, it was sometimes difficult to obtain enough nuclei with direct fixation.

Experiment III: Different Fixatives

In this experiment, three fixatives, Carnoy's, 75% ethanol, and 10% formalin, were tested, using gill tissues from diploids, with

TABLE 2.

Peak position and CV (mean ± SD) from FCM histograms of gill tissue of diploid Pacific oysters preserved in Carnoy's after different pre-treatments for 5–30 min (n = 5).

Peak	Pre-treatment time (min)	0.075 M KCl	De-ionized water	0.5% Triton-100	Direct fixation	Freeze control
Position	0				55.4 ± 1.2	54.2 ± 0.6
	5		50.6 ± 2.2			
	10	56.6 ± 1.7	52.7 ± 2.4	50.7 ± 3.8		
	15	56.6 ± 2.3	51.6 ± 3.9	50.9 ± 3.6		
	20	54.5 ± 1.8	52.1 ± 2.9	49.6 ± 2.0		
	25	55.8 ± 1.4	50.4 ± 2.4	49.9 ± 4.6		
	30	56.4 ± 1.0	52.5 ± 0.6	48.4 ± 3.9		
	ANOVA P value	0.361	0.717	0.822		
	Overall mean	55.98	51.64	49.90	55.42	54.20
	t test P value	0.642	0.226	0.007	0.928	
CV	0				11.7 ± 1.2	9.7 ± 0.7
	5		12.2 ± 2.6			
	10	12.0 ± 1.4	11.1 ± 0.9	13.5 ± 2.2		
	15	13.1 ± 2.6	11.3 ± 1.4	12.7 ± 2.3		
	20	12.6 ± 2.2	11.3 ± 1.5	13.3 ± 1.3		
	25	12.1 ± 1.8	11.6 ± 1.5	13.0 ± 2.3		
	30	11.6 ± 1.8	12.2 ± 1.0	13.6 ± 3.7		
	ANOVA P value	0.573	0.861	0.979		
	Overall mean	12.27	11.60	13.23	11.70	9.66
	t test P value	0.282	0.359	0.011	0.561	

TABLE 3.

Peak position and CV (means ± SD) from FCM hitograms of gill tissue of diploid Pacific oysters preserved in different fixatives after pre-treatment with 0.075 M KCl fur 5–20 min (n = 3).

Peak	Pre-treatment time (min)	Carnoy	Ethanol (75%)	Formalin (10%)	Freeze control
Position	0	—	—	—	53.3
	5	52.7 ± 2.0	53.3 ± 2.1	44.5 ± 5.1	—
	10	51.5 ± 3.7	52.2 ± 1.2	44.3 ± 1.5	—
	15	52.0 ± 0.9	52.3 ± 1.6	45.7 ± 2.3	—
	20	52.9 ± 1.8	51.8 ± 1.8	47.3 ± 2.6	—
	ANOVA P value	0.873	0.721	0.674	
	Overall mean	52.28	52.41	45.45	53.34
	t test P value	0.881	0.915	<0.001	
CV	0	—	—	—	9.9
	5	12.7 ± 0.6	10.5 ± 0.1	12.8 ± 1.0	—
	10	12.2 ± 1.3	9.8 ± 1.6	14.9 ± 1.3	—
	15	13.9 ± 2.8	9.9 ± 0.4	14.5 ± 0.6	—
	20	10.8 ± 0.9	9.2 ± 0.4	13.2 ± 0.7	—
	ANOVA P value	0.179	0.277	0.090	
	Overall mean	12.42	9.86	13.86	9.88
	t test P value	0.026	1.000	<0.001	

pre-treatments with 0.075 M KCl for 5, 10, 15, or 20 min. Again, the duration of hypotonic treatment did not affect peak position and CV for all three fixatives (Table 3). Of the three fixatives, only formalin affected FCM and produced significantly lower peak positions and higher CVs. Peak positions from Carnoy's and 75% ethanol fixation were unchanged relative to the control method. FCM histograms from ethanol-fixed samples were comparable in quality to that from the control method (Figure 1).

DISCUSSION

FCM is a powerful technique for ploidy determination in shellfish (Allen 1983, Chaiton and Allen 1985, Guo et al. 1993), and there is an increasing demand for ploidy analysis in shellfish research and production. Access to FCM for most shellfish labs and hatcheries is limited by difficulties in the storage and transportation of fresh or frozen samples. We had several experiences where important FCM samples degraded during shipment. We have previously attempted direct fixation with Carnoy's solution without success. The problem is that cells are tightly packed in fixed tissues, and nuclei cannot be easily dissociated. While there are protocols for nucleus isolation from fixed materials for clinical applications, they are too laborous for rapid ploidy analysis of large numbers of samples.

There are several ways to dissociate and isolate nuclei from fresh and fixed tissues, including mechanical, enzymatic, and chemical treatments (Song and Li 1992, Hedley 1994). Mechanical treatments such as freeze-and-thaw, vortexing, and aspirating are commonly used for dissociation of nuclei and found to be helpful in this study. Detergents can dissolve cell membrane and other structural components and disintegrate cells or nuclei from tissues (Vindelov 1977). In this study the detergent Triton X-100 was used to treat gill before fixation. Although it was effective in free-up nuclei, Triton X-100 reduced the peak position and increased the CV of treated samples. We did not test enzyme treatments as described in most clinical protocols because we wanted to develop a simple and inexpensive protocol.

As expected, direct fixation of oyster tissue samples made nucleus isolation difficult. Directly fixed samples often failed to produce enough nuclei for a normal FCM run (at 10,000 counts). It also affected the quality of FCM histograms with increased CVs. Direct fixation had no effects on peak position. We found that pre-treatments before fixation made nucleus isolation easier. Samples pre-treated with hypotonic solutions consistently produced sufficient numbers of nuclei. The hypotonic pre-treatment had no effects on the peak quality or CV. Pre-treatment affected peak position in two cases in Experiment I, which was not ob-

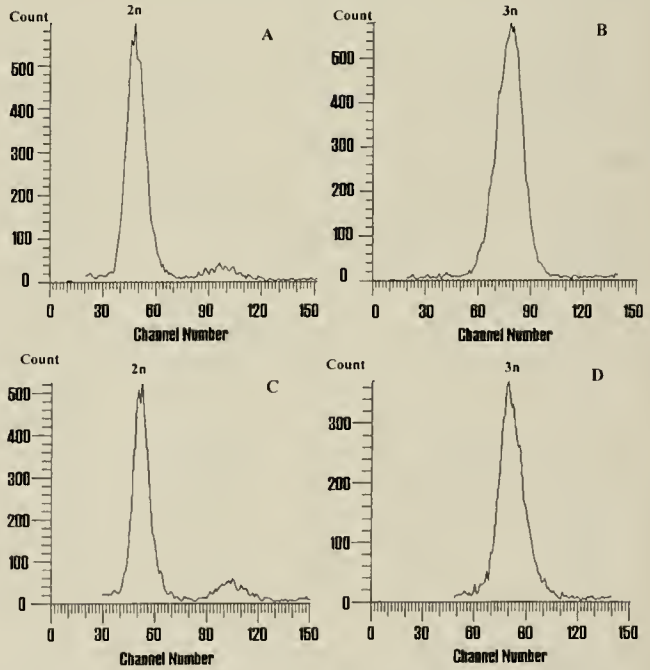

Figure 1. FCM histograms of diploid and triploid Pacific oysters, *Crassostrea gigas*. (A) diploid and (B) triploid: gill tissue frozen in DAPI/DMSO until analysis. (C) diploid and (D) triploid: gill tissues fixed in 75% ethanol and then stained with DAPI/DMSO.

served in subsequent experiments and was probably accidental. The level of variation in peak position observed does not affect ploidy determination. Because of the increased nucleus yield, we recommend hypotonic treatment before fixation.

Fixation is a process by which tissues or their components are fixed selectively at a particular stage to a desired state. The purpose of fixation is to kill the tissue without causing much damage or distortion of the components to be studied, such as nuclei for FCM. Formalin has the ability to fix DNA through reacting with related proteins (Sharma and Sharma 1980). It has been used to fix fish erythrocytes to prepare FCM samples (Crissman et al. 1979). Also, it has been used in preparing shellfish FCM samples (Allen 1983) and yield acceptable cell suspension. In this study fixation in formalin did not produce enough nuclei even with a hypotonic pre-treatment. The peak position of formalin fixed samples shifted lower and the CV was higher than that with the control method. Our results suggest that 10% formalin is not a good fixative for preserving oyster gill for FCM.

Both ethanol (75%) and Carnoy's fixative are acceptable for preserving gill tissue for FCM. FCM histograms from ethanol fixed samples were often the best with smallest CVs. Ethanol is more readily available, more stable, and less corrosive than Carnoy's fixative. Therefore, we recommend 75% ethanol over Carnoy's fixative for preserving tissues for FCM.

Results of this study show that certain tissues are better suited for FCM than others. Mantle was the most difficult tissue in providing enough nuclei for FCM. Hemolymph, while acceptable when frozen, significantly changed peak positions after fixation in Carnoy's fixative. The best tissue for FCM, fixed or frozen, was gill. It consistently produced large numbers of nuclei, which confirms our early experiences.

In summary, oyster tissue samples can be fixed for FCM analysis. With pre-treatments aimed at nucleus dissociation, fixation does not affect the quality of FCM and ploidy determination. With several factors considered, we recommend the follow protocol for fixing oyster samples for FCM: dissect a piece of gill tissue (~0.5 cm^2), treat with 0.075 M KCl for 10 min, cut into multiple pieces, fix in 75% ethanol, and change fixative once. The fixed sample can be stored at 4 °C or room temperature and transported at ambient temperature. Before FCM, the sample is washed once using phosphate-buffered saline (pH = 6.8) and transferred to a staining/isolation solution with 10% DMSO. The sample is frozen-and-thawed, votexed for 10 to 15 sec, aspirated 5 times with a 25-guage syringe, and then filtered through a 25-μm nytex screen. Although we did not test long-term storage, we were able to analyze samples stored at room temperature for 6 mo. This protocol has worked for adult zhikong scallop, *Chlamys farreri*, and oyster and scallop larvae, and it is probably applicable to most molluscs.

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EVALUATION OF A GLUCOSE OXIDASE/PEROXIDASE METHOD FOR INDIRECT MEASUREMENT OF GLYCOGEN CONTENT IN OYSTERS (*CRASSOSTREA VIRGINICA*)

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ABSTRACT A colorimetric method for indirect measurement of glycogen concentrations in tissue homogenates of eastern oysters (*Crassostrea virginica*) was evaluated. This method uses a conversion of glycogen to glucose by amyloglucosidase. The procedure was optimized for extracting buffer pH (5.0) and amyloglucosidase concentration (5 mg/mL). Coefficients of variation ($n = 10$) for oyster homogenates with mean glycogen concentrations of 84 and 242 mg/dL had within-run values of 3.29 and 3.66%, and between-run results of 4.46 and 3.15%, respectively. When mean glycogen concentrations of thawed oyster homogenates were compared with those of initial fresh homogenates, no significant ($P \leq 0.05$) differences were detected in samples thawed after 1 h, 1 day, 1 wk, or 1 mo. Glycogen recovery percentages of 104.1, 103.7, and 104.5% were obtained with mixed solutions containing 111, 94, and 19 mg/dL glycogen, respectively. The lower limit of sensitivity for the procedure was approximately 14 mg/dL. The assay was considered to be linear to 436 mg/dL. Lyophilized samples appeared to provide the most reliable determination of glycogen concentrations per gram of tissue by avoiding variable water content in oyster tissues. Initial laboratory ranges for tissue glycogen based on wet (mean ± 2 SD: 7–43 mg/g) and dry (mean ± 2 SD: 19–145 mg/g) weights were determined with 49 second-year growth oysters obtained during July 1998 (Covehead, Prince Edward Island, Canada). It was concluded that the colorimetric assay offered a reliable indication of tissue concentrations of glycogen in eastern oysters (*C. virginica*).

KEY WORDS: Glycogen, oysters, *Crassostrea virginica*, method validation, colorimetric analysis

INTRODUCTION

Glycogen in oyster tissues has been evaluated in association with growth (Kaufmann et al. 1994, Littlewood and Gordon 1988), nutrition level (Whyte et al. 1990), and reproductive cycle (Littlewood and Gordon 1988). Tissue concentrations of glycogen could impact on disease resistance and commercial shelf life in this species. Any method for glycogen determination in oysters must undergo a validation procedure to ensure accuracy of results. Validation procedures are mandatory prior to the use of any assay in a human or veterinary clinical chemistry laboratory (Peters and Westgard 1986, Murray et al. 1993). It is logical to use the same assurance of quality for assays used in laboratory investigations in shellfish. Validation includes assessment of precision, linearity, recovery, and sample stability for the reagent system (Peters and Westgard 1986). The method (Carr and Neff 1984) most frequently used to measure glycogen in shellfish tissues utilizes an enzymatic conversion of glycogen to glucose with amyloglucosidase, followed by measurement of glucose concentrations with commercially available reagents. This method was recently validated for *Mytilus edulis* tissues (Burton et al. 1997), but it would be erroneous to extrapolate results to other shellfish species. The purpose of the study reported here was to determine whether a commercial oxidase/peroxidase assay for measurement of glucose in human sera could be validated for indirect measurement of glycogen in homogenates of eastern oyster (*Crassostrea virginica*) tissues.

MATERIALS AND METHODS

Assay Procedure

Mature (6- to 7-cm shell length) oysters (*C. virginica*) were obtained from a lease in Covehead, Prince Edward Island, Canada. They were removed from their shells, blotted dry and weighed, then individually homogenized in ice cold trisodium citrate buffer. The assay procedure has been described previously for marine mussels (Burton et al. 1997), except that glycogen standards from oysters (Glycogen type II from oyster, Sigma Chemical Co., St. Louis, MO) rather than mussels were prepared. Briefly, following individual homogenization of oysters in buffer, the samples were heated in a boiling water bath, cooled, and rehomogenized. Aliquots were incubated with amyloglucosidase and the glucose content was determined using benchtop techniques and a commercial glucose reagent. Enzyme-untreated (blank) aliquots and standards were also prepared and analyzed using the same protocol.

Assay Optimization

A buffer volume of 100 mL for homogenization was chosen for ease of use and to minimize error due to sample loss. To determine whether the conversion of oyster glycogen to glucose could be optimized, final buffer pH values of 4.5, 5.0, and 5.5 were evaluated. To achieve this, six oysters were homogenized separately in 20 mL of 0.1 M trisodium citrate buffer (pH 5.0). After boiling water bath incubation and rehomogenization, the samples were divided into three aliquots of 5.0 mL each. To these aliquots, 20 mL of 0.1 M trisodium citrate buffer of varying pH were added to obtain final buffer pH values of 4.5, 5.0, and 5.5. The different aliquots were analyzed as described previously. To determine whether the concentration of amyloglucosidase could be optimized for maximal conversion of oyster glycogen to glucose, amyloglucosidase solutions with concentrations of 40, 20, 10, 5, 1, and 0.5 mg/mL were prepared in 0.1 M trisodium citrate buffer (pH 5.0). After the boiling water bath treatment and rehomogenization as described above, 5.0 mL aliquots of homogenate from six separate

oyster samples were each incubated with these amyloglucosidase solutions. Enzyme-untreated (blank) solutions and standards were prepared as previously described.

Assay Evaluation

To evaluate assay precision, within-run and between-run (day-to-day) studies were conducted, and coefficients of variation (CV) were calculated. Two oyster samples with mean glycogen concentrations of 84 and 242 mg/dL were analyzed 10 times to obtain data for the within-run calculations. Aliquots of the same homogenates were frozen, thawed, and analyzed 10 times in separate runs over a period of 1 month to obtain between-run precision. To determine the frozen stability of oyster glycogen samples, an additional six separate homogenates were analyzed for glycogen levels (time zero) and aliquots frozen at −25 °C. These were thawed after periods of 1 h, 1 day, 1 wk, and 1 mo, and the glycogen concentrations were determined.

Recovery experiments were conducted to ensure that oyster glycogen was being recovered and to evaluate the minimal converted glycogen that could be reliably measured by the assay. To accomplish this, oyster homogenates of known glycogen concentrations were diluted 1:9 with the commercial glucose standard (90 mg/dL) provided with the reagent kit, and the observed recoveries were compared to the theoretical amounts. For minimum recovery experiments, two oyster homogenates and 0.1 M trisodium citrate buffer (pH 5.0) were used. One homogenate was diluted (2-, 3-, and 4-fold) with the second. A second set of mixtures (blanks) was prepared using buffer and the second homogenate in the same ratios. Both sets of mixtures were analyzed, and the glycogen recovered was determined.

Linearity of the assay was evaluated by measuring the glycogen concentrations of a set of serial dilutions (in 0.1 M trisodium citrate buffer, pH 5.0) of an oyster homogenate sample with a high glycogen concentration (436 mg/dL). The dilutions, based on percentages of the previous sample in the series (with expected results in brackets expressed as mg/dL) were as follows: 100% (436), 75% (327), 66.7% (218), 50% (109), 50% (55), 50% (27), and 50% (14).

Initial laboratory ranges (mean ± 2 SD) for tissue glycogen based on both wet and dry weights was determined with 49 second-year growth oysters obtained during July 1998 from the Covehead region of Prince Edward Island, Canada. To express the glycogen amounts on a wet weight basis, the homogenates were aliquoted (11 mL) after the boiling water treatment and frozen at −25 °C for 2 weeks. After thawing, the aliquots were analyzed for glycogen content and expressed as mg/g wet weight (initial shucked weight). To obtain the glycogen amounts on a dry weight basis, a 20-mL aliquot of each oyster sample was obtained following the boiling water bath treatment and rehomogenization and was dispensed into a 50 mL serum bottle (Wheaton '400' borosilicate glass; Wheaton, Millville, NJ). Samples were then lyophilized in a freeze dryer (Labconco Corp., Kansas City, MO) and stored in a refrigerator (4 °C). The lyophilized sample was weighed (weight corrected for buffer salt content), reconstituted in 20 mL of deionized water and analyzed for glycogen content. The glycogen concentrations were expressed as mg/g dry tissue.

Statistical Analysis

A computer software program (Minitab Statistical Software Inc., Version 9.1, State College, PA) was used for statistical calculations. All tests were performed at the $P \leq 0.05$ significance level. Repeated-measures analysis of variance (ANOVA) calculations were performed to determine if differences existed in glycogen concentrations due to changes in buffer pH and for different amyloglucosidase concentrations. A repeated measures ANOVA was also performed to see if significant differences were present between glycogen concentrations of fresh and frozen aliquots in the frozen stability assessment over a 1-month period. A paired t-test was used to test for difference between glycogen concentration obtained with frozen (wet) and lyophilized (reconstituted) homogenates. Linear regression analysis was performed for comparison between glycogen concentrations of the wet and lyophilized (reconstituted) samples, expressed as either mg/g or mg/dL and between wet and dry tissue weights.

RESULTS

Varying the buffer pH and changing the concentration of amyloglucosidase in the reagent mixture resulted in no significant ($P \leq 0.05$) optimization in glycogen concentrations in oyster samples. The buffer pH of 5.0 and an amyloglucosidase concentration of 0.5% (5 mg/mL) previously reported by Carr and Neff (1984) and used for marine mussel tissues (Burton et al. 1997) were therefore used throughout the study.

In the precision study, the coefficients of variation ($n = 10$) for oyster homogenates with mean glycogen concentrations of 84 and 242 mg/dL had within-run values of 3.29 and 3.66%, and between-run results of 4.46 and 3.15%, respectively.

In the frozen stability assessment, the mean percentage differences in the glycogen concentrations of the thawed samples compared to the fresh samples was 1.3, 2.7, 1.6, and 1.4%, for the 1 h, 1 day, 1 wk, and 1 mo samples, respectively. No significant ($P \leq 0.05$) differences were observed between fresh aliquots and samples thawed after each time period. Therefore, glycogen in frozen homogenates was concluded to be stable for at least 1 month. Linearity was assessed to be at least 436 mg/dL (Fig. 1) as serial dilutions of an oyster homogenate solution with a high glycogen concentration (436 mg/dL) gave observed results within 3.7% of expected, including a value with an expected glycogen

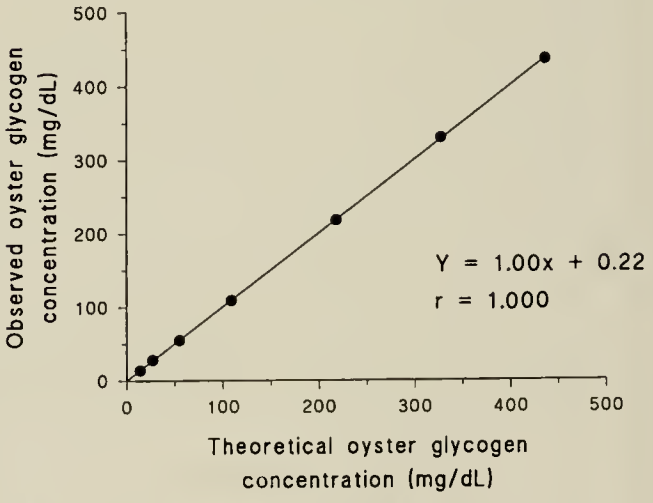

Figure 1. Linearity plot of a tissue homogenate of an oyster (*C. virginica*) with a high glycogen concentration (436 mg/dL) diluted in 0.1 M trisodium citrate buffer (pH 5.0). Observed glycogen concentration correlated closely (within 3.7%) with expected concentrations.

concentration of 14 mg/dL. Recovery percentages of 103.7% and 104.1% were obtained using mixtures containing 94 and 111 mg/dL, respectively (Table 1). Concentrations of 25 and 19 mg/dL were acceptably recovered (94% and 104.5%, respectively) using the minimum recovery experiments. However, solutions containing an expected glycogen concentration of 13.3 mg/dL had an unacceptably low recovery of 27.1%. Based on the combined information from the minimum recovery experiment and the linearity assessment, the lower limit of sensitivity for this assay was considered to be approximately 14 mg/dL.

Initial laboratory ranges for oyster tissue glycogen concentrations are presented in Table 2. Supporting data regarding wet and dry weights for these 49 oysters are also provided. Linear regression analysis of the relationship between the converted glycogen concentration (expressed as mg/dL in solution) in wet and dry samples, showed significance ($P \leq 0.05$) with an r value of 0.98 (Fig. 2). Additionally, a paired t-test indicated no significant ($P \leq 0.05$) difference in these converted glycogen concentrations (in mg/dL) between wet and dry samples. When expressed as glycogen concentrations (in mg) per gram of wet or dry tissue, a significant ($P \leq 0.05$) relationship was found, with an acceptable r-value of 0.87 (Fig. 3). The relationship between the actual weights of the wet and dry tissues (in grams) was significant ($P \leq 0.05$), with an r-value of 0.65.

DISCUSSION

The colorimetric assay evaluated in this study was determined to be a reliable indirect indicator of tissue glycogen concentrations in eastern oysters (*C. virginica*).

Two experiments were carried out to determine whether the conversion of oyster glycogen to glucose could be optimized by changing the buffer pH and/or the amyloglucosidase concentration to levels other than previously published by Carr and Neff (1984) in soft shell clams (*Mya truncata*) and scallop adductor muscle (*Placopecten magellanecus*) or Burton et al. (1997) in mussels (*Mytilus edulis*). Unit changes in pH (4.5–5.5) and a 80-fold increase in amyloglucosidase (0.5–40 mg/mL) quantities resulted in no significant change in glycogen concentration. Therefore, previously reported values for pH and amyloglucosidase were used in the rest of the evaluation and are recommended for future glycogen assays with oyster tissue.

The precision of this assay is acceptable, with coefficients of variation of less than 3.7% and 4.5% for within-run and between-run evaluations, respectively. These values are slightly higher than those reported using the same technique in marine mussels (Burton et al. 1997). This may be attributed to variability in the technical skills of different laboratory personnel. The stability of the glycogen levels in the frozen aliquots indicates that samples could be frozen for at least one month, batched and analyzed in groups. If glycogen concentrations are proven to influence disease resistance and commercial shelf life in future studies, batch analysis would be cost-effective and desirable from a marketing viewpoint. Recovery experiments indicate that the oyster glucose produced after amyloglucosidase conversion responds in the assay identically to human glucose. Linearity of the system is excellent between 436 and

TABLE 1.

Recovery data for a colorimetric method for indirect glycogen measurement in oysters (*Crassostrea virginica*)

Sample Description	Expected Glycogen (mg/dL)	Observed Glycogen (mg/dL)	Recovery Percentages*
Mixture 1	90.3	93.7	103.7
Mixture 2	106.8	111.3	104.1
Mixture 3	26.6	25.0	94.0
Mixture 4	17.7	18.5	104.5
Mixture 5	13.3	3.6	27.1

A = oyster tissue homogenate (93.2 mg/dL), B = commercial glucose standard (90 mg/dL), C = oyster tissue homogenate (258 mg/dL), D = oyster tissue homogenate (53.1 mg/dL), E = 0.1 M trisodium citrate buffer (pH 5.0).

Mixture 1 = (1 volume A + 9 volumes B), Mixture 2 = (1 volume C + 9 volumes B), Mixture 3 = (1 volume D + 1 volume C) – (1 volume E + 1 volume C), Mixture 4 = (1 volume D + 2 volumes C) – (1 volume E + 2 volumes C), Mixture 5 = (1 volume D + 3 volumes C) – (1 volume E + 3 volumes C).

* Recovery percentages = (observed concentration)/(expected concentration) × 100.

TABLE 2.

Laboratory ranges for oyster tissue glycogen based upon wet and dry weights for 49 second-year growth oysters (*Crassostrea virginica*) obtained during July 1998 from the Covehead region of Prince Edward Island, Canada

Variable	Mean	Standard Deviation (SD)	Range (mean ± 2 SD)
Wet weight (g)	2.63	0.55	1.53–3.73
Dry weight (g)	0.80	0.14	0.52–1.08
Glycogen (mg/dL), wet samples	65.6	27.8	10–121
Glycogen (mg/dL), lyophilized tissue	66.9	28.8	9–125
Glycogen (mg/g), wet weight	24.8	9.04	7–43
Glycogen (mg/g), dry weight	82.1	31.5	19–145

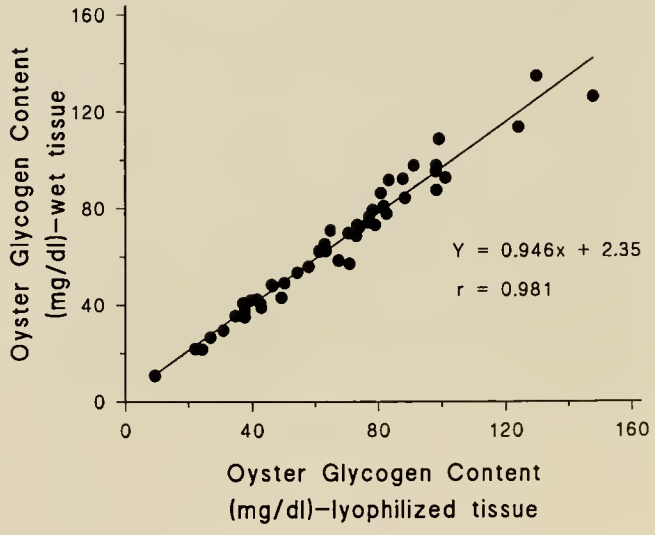

Figure 2. Correlation and linear regression analysis of glycogen concentrations (mg/dL in solution) for 49 oysters (*C. virginica*) obtained from the Covehead region, Prince Edward Island, Canada in July 1998. Glycogen concentrations were obtained with wet and lyophilized samples and have a significant ($P \leq 0.05$) correlation.

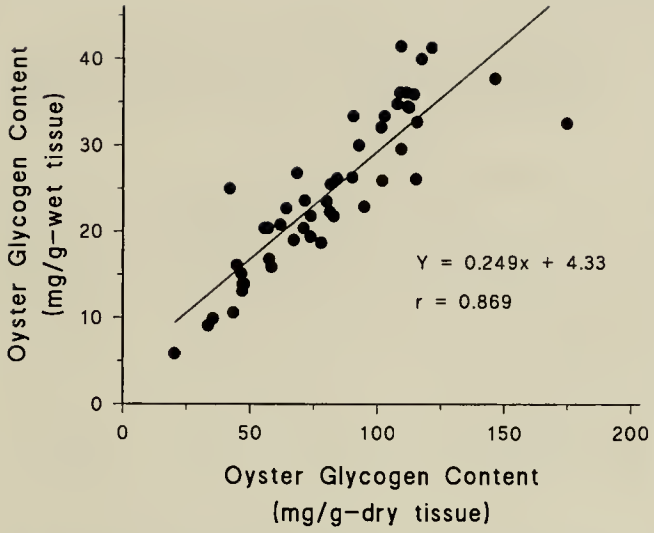

Figure 3. Regression analysis plot comparing glycogen concentrations of 49 oysters (*C. virginica*) in July 1998 from the Covehead region, Prince Edward Island, Canada. Glycogen concentrations are expressed as mg glycogen/g-wet oyster weight and mg glycogen/g-dry weight (lyophilized). A significant ($P \leq 0.05$) correlation was observed.

14 mg/dL (Fig. 1) with results from diluted samples within 3.7% of expected concentrations. Minimal recovery experiments combined with linearity assessment suggest that 14 mg/dL represents the lower limit of sensitivity for the assay.

Comparison of the converted glycogen concentrations of the wet and dry samples (expressed as mg/dL in solution), was performed, using both regression analysis and a paired *t* test. The relationship between these converted glycogen concentrations in wet and dry samples was significant, with a high correlation coefficient of 0.98. The paired t-test also indicated no significant difference in these converted glycogen concentrations (mg/dL) between wet and lyophilized homogenates. These results suggest that lyophilization, as might be expected, does not affect the glycogen levels in the tissue samples.

Correlation results for the glycogen concentration of oysters expressed as milligrams of glycogen per gram of wet or dry tissue (Fig. 3) was significant and had an acceptable correlation coefficient of 0.87. The correlation between the actual weights of these wet and dry samples, while statistically significant, had a much lower (0.65) correlation coefficient. As careful surface blotting was done, this probably reflects variable water content within the tissues of the wet weight samples. Overall, these results suggest that the most accurate values are obtained with glycogen expressed on a lyophilized dry weight basis. However, glycogen expressed on a shucked wet weight basis could still be useful to commercial growers desiring quick, albeit less accurate results, for assessments such as shelf life prediction. A shucked wet weight glycogen analysis can be performed in less than 24 h compared to 4–5 days required for the dry weight determination. In this study, it was convenient to process samples at room temperature over a 24-h period. Carr and Neff (1984) report that samples can also be incubated in a water bath (55 °C) for 2 h. This would appreciably shorten analysis time in a commercial setting.

It should be noted that the boiling water bath incubation represents an important step in the procedure. Heating has been reported (Carr and Neff 1984) to inactivate endogenous glycogenases in shellfish tissues, which could alter the glycogen concentration obtained using enzymatic glucose analysis. More convenient methods of heating samples, such as microwave use, remain to be evaluated.

Initial laboratory range (Table 2) for tissue glycogen based on both wet and dry weights was determined for samples from the Covehead region of Prince Edward Island in July, 1998. More work is required to derive reliable reference ranges for whole-body glycogen concentrations in oysters for time of year, reproductive activity and geographic region. In summary, indirect tissue glycogen concentrations in the oyster (*C. virginica*) were reliably determined by this colorimetric assay . With this assurance of reliability, further studies may show tissue glycogen concentration to be valuable in assessing overall health status or in predicting shelf life in eastern oysters.

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SYNCHRONOUS OOGENESIS DURING THE SEMILUNAR SPAWNING CYCLE OF THE TROPICAL ABALONE *HALIOTIS ASININA*

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ABSTRACT On the southern Great Barrier Reef, *Haliotis asinina* (Vetigastropoda: Pleurotomarioidea) synchronously spawn every 2 wk in a predictable fashion, allowing detailed analysis of reproduction, gametogenesis, and gonad development. Histological examination of the ovaries of members of the Heron Reef population during this semilunar cycle reveals that oogenesis is also synchronous and predictable, and requires more than two spawning cycles (i.e. >28 days) to complete. Shortly after a spawning event the ovary comprises two cohorts of primary oocytes, one of which will be released at the next spawning event, and clusters of oogonia. At this time there is a rapid proliferation and expansion of trabeculae, germinal epithelial, and oogonia, and a dramatic increase in the size of the vitellogenic oocytes to be spawned at the next spawning event. Within 4 days these oocytes have filled the ovary. On the day of the next spawning a lumen forms in the ovary as a result of localized degradation of trabeculae. The large primary oocytes dissociate from the receding trabeculae, initiate maturation, and accumulate in the lumen; these oocytes become embedded in a jelly coat layer. The next cohort of oocytes remain attached to the trabeculae. The jelly coat appears to be completely dissolved within 30 min of spawning. Comparison of the oogenesis and ovary development in *H. asinina* with other abalone species indicates that these processes are very similar in tropical and temperate abalone. This suggests that insights into the regulation of reproduction and spawning in *H. asinina* are likely to be applicable to other haliotids.

KEY WORDS: Gametogenesis, gastropod, germinal vesicle, haliotid, spawning cycle

INTRODUCTION

Reproductive cycles of marine invertebrates are often regulated by a combination of exogenous and endogenous rhythms (Olive and Garwood 1983). Temperature is often suggested as the main environmental variable regulating abalone reproductive cycles (Hahn 1989, Wells and Keesing 1989). Gametogenesis in the Japanese abalone *Haliotis discus hannai* has been linked quantitatively with temperature. This species must experience a critical minimum water temperature before gonad maturation is initiated (Kikuchi and Uki 1974). Gonad maturation increases linearly with increasing water temperature and the stage of maturity can be predicted by the time spent above the critical minimum temperature (Uki and Kikuchi 1984).

Reproductive cycles of different species and populations of abalone vary significantly (Webber and Giese 1969, Shepherd and Laws 1974, Hahn 1989, Hooker and Creese 1995, Wilson and Schiel 1995). For example, the five species of abalone that inhabit the waters along the southern Australian coast either spawn synchronously during spring and summer (*H. cyclobates* and *H. laevigata*), autumn and winter (*H. ruber*), or intermittently throughout the year (*H. roei* and *H. scalaris*) (Shepherd and Laws 1974). Webber and Giese (1969) observed that two populations of the black abalone *H. cracherodii*, 11 km apart, spawn at different times. This high degree of inter- and intraspecific variation suggests that both intrinsic species-specific and local environmental factors are important in regulating reproduction.

H. asinina, the largest of the tropical abalone species, is distributed throughout the coral reefs of the Indo-Pacific, including the Great Barrier Reef (Talmadge 1963, Fallu 1991). In The Philippines, *H. asinina* are serial spawners, spawning asynchronously year-round except during May and June (Capinpin et al. 1998). In Thailand, the spawning season of *H. asinina* is year-round except April and May, and peaks in October and November when the water temperature is lowest (Singhagraiwan and Doi 1992). Both Capinpin et al. (1998) and Singhagraiwan and Doi (1992) restricted their observations almost solely to *H. asinina* that were housed in aquaria for months or ones that had been bred in captivity. Capinpin et al. (1998) did observe that recently captured *H. asinina* (less than 6 wk) exhibited spawning patterns that were more synchronous than longer-term captive abalone. *H. asinina* on Heron Reef, Southern Great Barrier Reef exhibit synchronous semilunar spawnings from October to April (Counihan et al. in press). This highly predictable and rapid spawning cycle appears to be unique amongst the haliotids and facilitates the analysis of reproduction, gametogenesis, and gonad development, and the exogenous and intrinsic factors regulating these processes.

As a first step toward understanding gametogenesis and spawning in *H. asinina* we have undertaken a histological examination of female ovaries during the semilunar spawning cycle. Previous studies on the reproductive biology and gametogenesis in *H. asinina* have focused either upon determining the reproductive status of wild-caught abalone (Capinpin et al. 1998) or classification of gonads and gametes (Apisawetakan et al. 1997). Here we report that oogenesis and ovary development in Heron Reef *H. asinina* is synchronous, predictable, and tightly linked to the semilunar spawning cycle. The different oocyte stages present within the ovary can be classified into one of three cohorts that are developing simultaneously in the ovary. These three cohorts will be spawned sequentially over the next three spawning events. These data demonstrate that while a single spawning cycle in *H. asinina* is extremely short, being 13 to 15 days long, oogenesis requires more than two spawning cycles (i.e. >28 days) to complete.

MATERIALS AND METHODS

H. asinina were collected from the outer-coral algal subzone of the reef flat of Heron Reef (23°27′S, 151°55′E) and kept at The

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University of Queensland Heron Island Research Station. Captured abalone were maintained in flowing, ambient seawater and a natural light regime, and fed *Gracilaria* spp. every 3 days. Between November 4 and December 10, 1995, ovaries were excised from female abalone daily. These were washed in 0.2 μm filtered seawater, left in Bouin's fixative for 6 days, washed three times in 70% ethanol over 3 days, dehydrated through a graded ethanol series into xylene, and embedded in paraffin wax. Eight-millimeter-thick transverse sections were cut at four equally partitioned sites along the ovary. Sections were transferred to glass microscope slides, dried at 40 °C overnight, and stained with Mayer's haematoxylin and eosin. Spawned eggs were fertilized as described in Counihan et al. (1998). Live eggs, zygotes, and embryos were whole mounted under raised coverslips. All light micrographs were taken with an Olympus BX50 microscope with differential interference contrast optics.

RESULTS

The 36-day series of sections taken from four equally spaced regions of the *H. asinina* ovary revealed that oogenesis within an individual female proceeded synchronously throughout the ovary. Hence a section through any region of the ovary was representative of the developmental state of the entire gonad. Ovaries that were isolated from different females on the same day (i.e. at the same time of the spawning cycle) were structurally very similar and possessed oocytes at the same stage of oogenesis.

In a sexually mature females the green ovaries surround the hepatopancreas, forming the conical appendage. Non-invasive analysis of the conical appendage revealed that the extent of coverage of the digestive gland by the ovary varied with season and time in the semilunar spawning cycle (Singhagraiwan and Doi 1992, Capinpin et al. 1998). Microscopic analysis of the ovary indicated that gross morphological changes in the conical appendage during the spawning cycle correlated with changes in the structure of the ovary and the developmental state of oocytes within (Figs. 1 and 2).

Oogenesis and ovary development were monitored during the semilunar spawning cycle of *H. asinina* in November and December, 1995. During this time of the reproductive season nearly all sexually mature individuals were ripe and spawning regularly (Counihan et al. in press). Inspection of ovaries that were fixed during the spawning event revealed that three cohorts of oocytes were present (Fig. 1, A and B). These were classified according to developmental stage, with (1) oocytes that were in process of being spawned being classified as mature oocytes (i.e. undergone germinal vesicle breakdown); (2) large, late vitellogenic primary oocytes attached to the trabeculae being classified as Cohort I oocytes; and (3) small, early/pre-vitellogenic primary oocytes attached to the trabeculae being classified as Cohort II oocytes. Clusters of small (8–15 μm) oogonia tightly associated with the trabecular connective tissue were also present (Figs. 1 and 2). At spawning, ovary structure varied within individual females, with trabeculae in most of the gonad projecting partially into the center of the ovary from both proximal (side adjacent to the digestive gland) and distal (side adjacent to the epidermis) sides; the central portion of this part of the gonad lacked trabeculae (Fig. 2).

Toward the posterior tip of the ovary, trabeculae traversed the entire gonad from the epidermal to the digestive gland side (Fig. 1A). Cohort I and II oocytes and oogonia were attached to the trabeculae and interspersed amongst each other (Fig. 1, A–D). The base of Cohort I oocytes (i.e. side attached to the trabeculae) corresponds to the future vegetal side, as the germinal vesicle (GV) was located at the opposite end of the developing oocyte (Fig. 1B). Both Cohort I and II primary oocytes had intact GVs and nucleoli, suggesting these oocytes were actively transcribing rRNA genes. A single nucleolus was observed per GV, suggesting that single nucleolar organizing region (i.e. rRNA gene cluster) was present in the genome of *H. asinina* (Degnan et al. 1990). The GV remained intact until the day of the spawning, at which time mature oocytes became dissociated from the trabeculae and predominantly localized to the central cavity or oviduct (Figs. 1A and 2). These oocytes had undergone germinal vesicle breakdown (GVBD) and had initiated meiosis to first metaphase.

The next morning approximately 8 h after spawning had finished, most of the trabeculae had increased so as to span most of the ovary from proximal to distal sides (i.e. the lumen had largely disappeared; Fig 1. C and D). We did not determine if this apparent growth was because of cell proliferation in the trabeculae (i.e. regeneration), changes in the shape of this tissue, or a combination of these processes. Cohort I and II oocytes and oogonia remained associated with the trabeculae and spanned its entire length, suggesting the structural modification of the trabeculae was partially responsible for reorganization of the gonad. Active rRNA gene transcription was detected in both cohorts of oocyte at this stage of gonad development. There appeared to be little increase in size of Cohort I and II oocytes at this stage.

A small number of unspawned mature oocytes were present in the gonad after the spawning event had finished (Fig. 1, C and E). At 8 h after spawning these oocytes were being engulfed by small amoebocytic cells (Fig. 1E). These cells were particularly abundant in the jelly coat and appeared to phagocytize this acellular material (Fig. 1E). By 2 d after spawning we did not detect mature oocytes or amoebocytes in the gonad, suggesting that the oocytes had degenerated and been phagocytized by the amoebocytes, which had subsequently migrated from the gonad. At this stage, the distance between rows of trabeculae appeared to have decreased and the trabeculae had become thicker (Fig. 1F). The yolk content in Cohort I primary oocytes had increased slightly. These oocytes had follicular cells surrounding the base (Fig. 1G).

Fours days after the previous spawning, the ovary was packed with yolky, polygonal Cohort I primary oocytes (Fig. 1, H and I). Cohort II oocytes were present interspersed amongst the large Cohort I oocytes; oogonia were not readily observed in the sectioned ovaries. The base of Cohort I oocytes that was attached to the trabeculae had widened, giving the oocyte its polygonal shape (Fig. 1I). A thin vitelline envelope or layer (5–10 μm thick) and jelly coat were first observed around the Cohort I oocytes at this stage. Between 4 and 13 d post-spawning stages we did not detect any obvious differences in the size and shape of the Cohort I gonads oocytes or in the overall structure of the gonad, except that the vitelline envelopes and jelly coats were expanded in the older oocytes (Fig. 1, J–L).

Twelve to 18 h before the next spawning, the gonad and Cohort I oocytes began undergoing dramatic changes (Fig. 2, A and B). Trabeculae became thin and no longer traversed the ovary from the proximal and distal edges. Trabeculae first disappeared from the middle of the ovary (Fig. 2A) and appeared to be receding from the center of the ovary towards the periphery. Amoebocytic cells were not associated with the tip of the receding connective tissue, which increased in thickness towards the periphery (Fig. 2B). With an increase in the thickness of the peripheral trabeculae, muscle cells

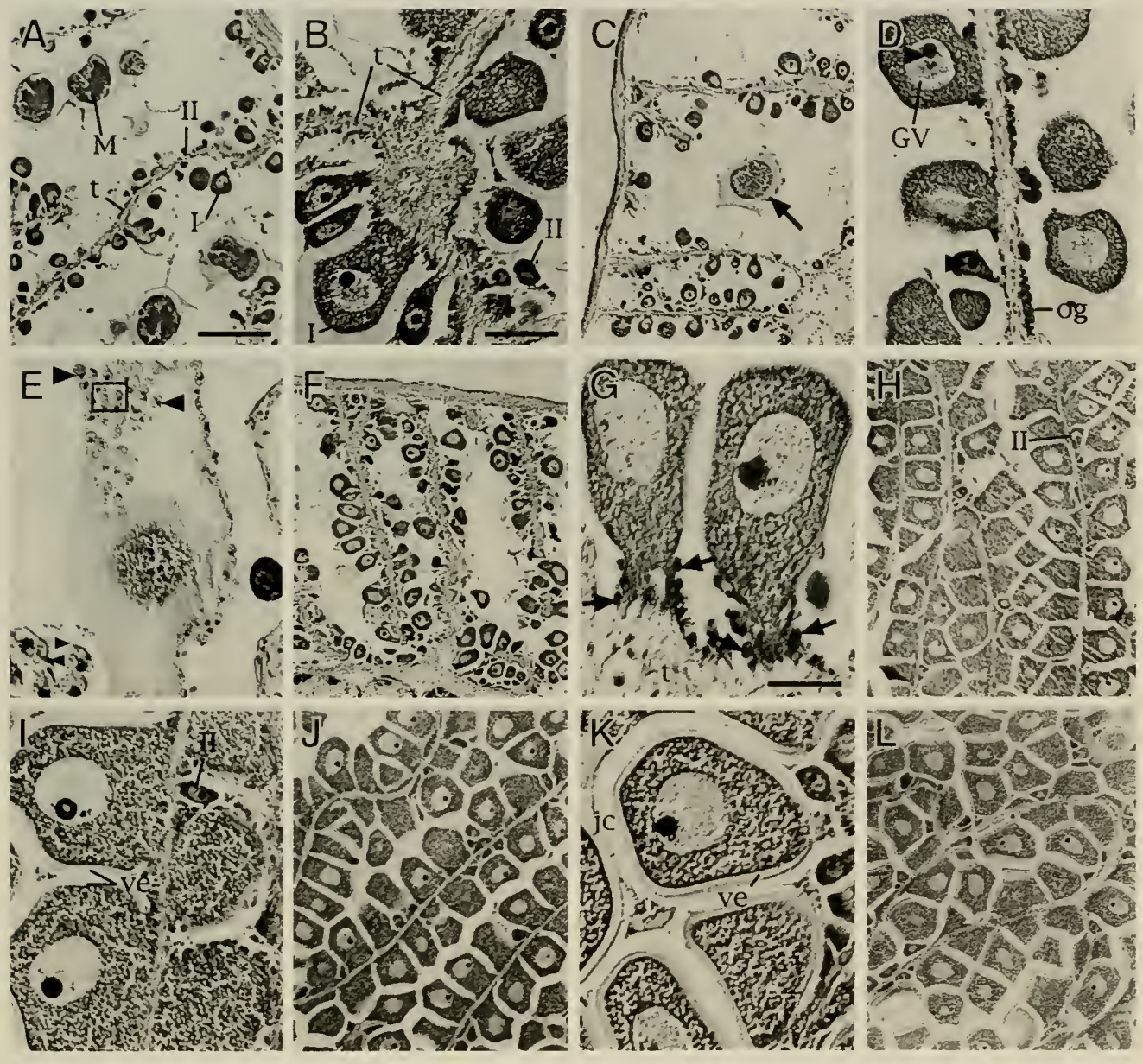

Figure 1. Oogenesis and ovary development during the semilunar spawning cycle of *H. asinina*. Part 1, vitellogenesis. All photomicrographs are haematoxylin-eosin stained transverse sections through the abalone ovary. A and B) Mid-spawn. A section of the ovary where there is limited trabecular degradation (i.e. where *de novo* oviduct formation has not occurred). Mature oocytes (M) are dissociated from trabeculae (t) while Cohort I and II primary oocytes are attached. C–E) 8 h after spawning is completed. C and D) Trabeculae traverse almost the entire ovary. Active rRNA gene transcription is occurring in the germinal vesicles (GV) of attached Cohort I and II oocytes as detected by the presence of nucleoli (arrowheads). A small number of unspawned mature oocytes are present (arrow in C). Clusters of oogonia (og) are present on the trabeculae. E) An unspawned oocyte with multiple small amoeboid cells in the jelly coat (arrowheads); these cells appear to be phagocytizing the jelly coat. The micrograph in the bottom left corresponds to the box; many of the amoeboid cells have pseudopodia (arrowheads). F and G) 2 days post-spawning. The distance between rows of trabeculae decreases and the trabeculae have become thicker and more muscular. The yolk content in Cohort I primary oocytes has increased. Follicular cells (arrows on some cells) surround the base of the oocyte. Unspawned mature oocytes are never detected in this or subsequent stages. H and I) 4 days post-spawn. The ovary is packed with yolky Cohort I primary oocytes; small Cohort II oocytes and trabeculae are still evident, although thinner than 2 days before. The vitelline envelope (ve) surrounding Cohort I oocytes is approximately 5- to 10-μm thick. J and K) 10 days post-spawning. There is no obvious differences between 4 and 10 days post-spawning ovaries or Cohort I oocytes, except the vitelline envelope is thicker, being typically 10- to 15-μm wide in the older oocytes and jelly coat (jc) later is obvious. L) 12 days post-spawning (2 days before next spawning). Cohort I primary oocytes are associated with trabeculae and are approximately the same size and possess the same histological characters as those in 4 and 10 post-spawning ovaries (see Fig. 1, H–K), except the vitelline envelope around the oocytes is thicker (approximately 12–17-μm thick). Scale bars: A, C, F, H, J, and L, 200 μm; B, D, E, I, and K, 50 μm; G, 25 μm.

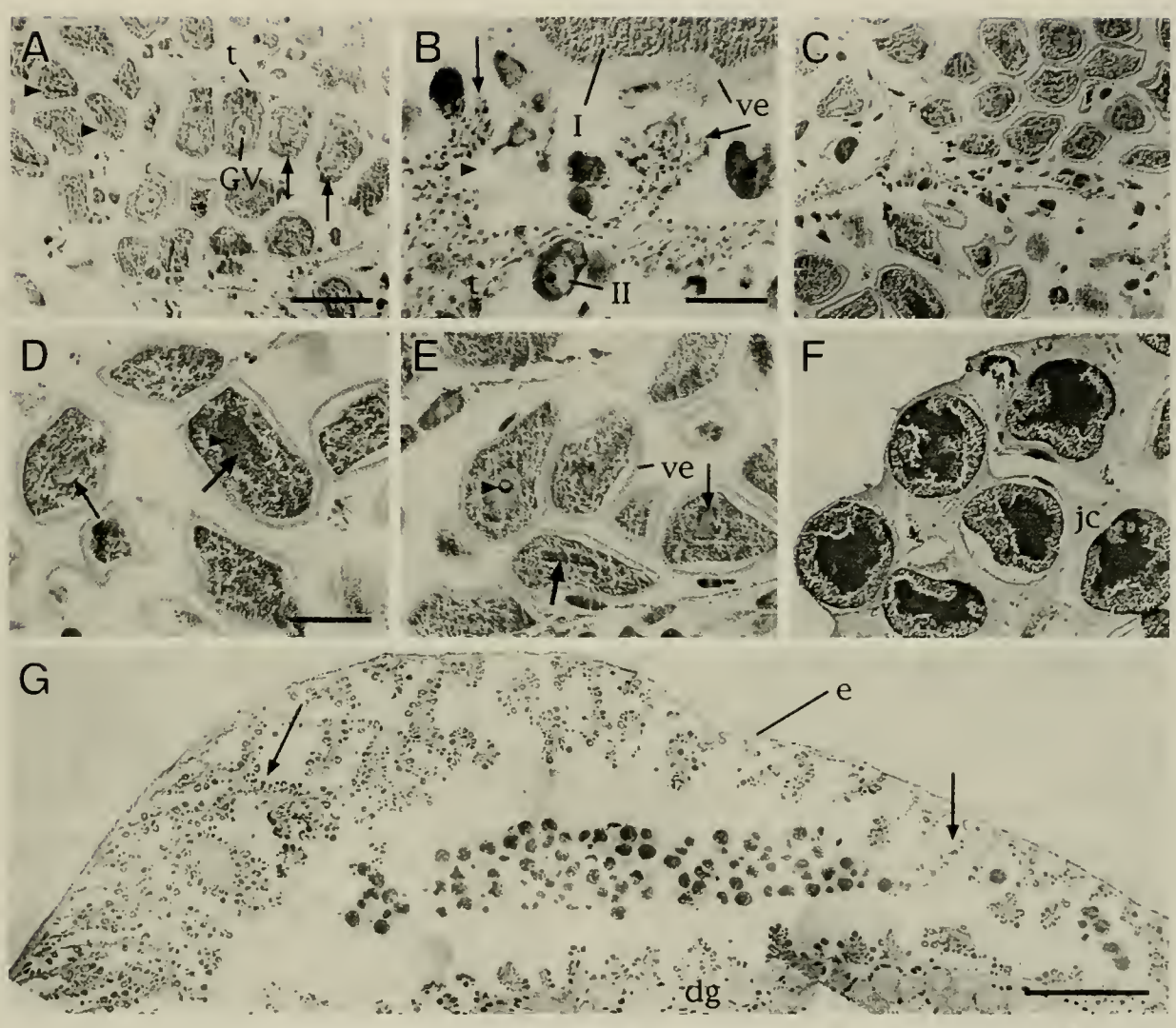

Figure 2. Oogenesis and ovary development during the semilunar spawning cycle of *H. asinina*. Part II, trabeculae breakdown, oocyte maturation, and *de novo* oviduct formation. All photomicrographs are haematoxylin-eosin stained transverse sections through the abalone ovary. A and B) 12 h before spawning. A) Trabeculae (t) are disappearing from the middle of the ovary. Cohort I oocytes are either dissociated from or attached to trabeculae, depending on location within the ovary; peripheral oocytes appear to be still attached (arrows). Oocytes associated with trabeculae appear to still have intact germinal vesicles (GV) with nucleoli, while a majority of released oocytes appear to be in the process of maturation and germinal vesicle breakdown (arrowheads). B) Higher magnification of the region where trabeculae appears to be degrading or retracting (arrows) with Cohort I and II oocytes; jc, jelly coat. C–E) 4 to 6 h before spawning. Trabeculae degeneration continues with further release of oocytes. Oocyte maturation is characterized first by the loss of the nucleolar organizing region (arrowheads), then by the absence of a distinct germinal vesicle membrane (i.e. nuclear envelope; small arrows), and finally by a change in staining of nuclear material from light to dark purple (large arrows). At this stage vitelline envelopes appear to be separating from the surfaces of the oocytes. F) Mature oocytes at spawning. Oocytes are embedded in a common jelly coat and have completed germinal vesicle breakdown. G) Ovary at spawning, with mature oocytes in a jelly coat mass in the newly formed oviduct. The digestive gland (dg) is at bottom and the outer epidermis (e) is at the top. Trabeculae with associated Cohort I (previously defined as Cohort II) and II oocytes project predominantly from the distal edge of the gonad (arrows). Scale bars: A and C, 200 μm; B, 50 μm; D, E,and F, 100 μm; G, 1 mm.

became apparent (Fig. 2B). Cohort I oocytes were either dissociated from or attached to trabeculae, depending on their location within the ovary (Fig. 2A), with oocytes closer to the periphery of ovary largely attached to the trabeculae. Oocytes that were no longer attached to the trabeculae appeared to be undergoing maturation and GVBD, while attached oocytes maintained intact GVs with nucleoli. The first phase of GVBD was characterized by the degeneration of the nucleoli, which initially took on a perforated appearance. This was followed by the loss of a distinct GV membrane and a change in staining of nuclear material from light to dark purple (Fig. 2, A–E). At this stage the vitelline envelope appeared to become dissociated from the oocyte plasma membrane. Shortly before spawning the mature oocytes (previously Cohort I oocytes) coalesced in the center of the ovary and appeared to become embedded in a common jelly coat (Fig. 2, F and G). The vitelline envelope appeared to be much thinner in the mature oocytes compared to oocytes prior to maturation (Fig. 2, F and D). At spawning this mass of mature oocytes was released into the seawater, where they dissociated into individual eggs (Fig. 3).

The spawned eggs were surrounded by a vitelline envelope that was less than 1 μm thick. A perivitelline space was present between this envelope and the oocyte. Surrounding the entire egg

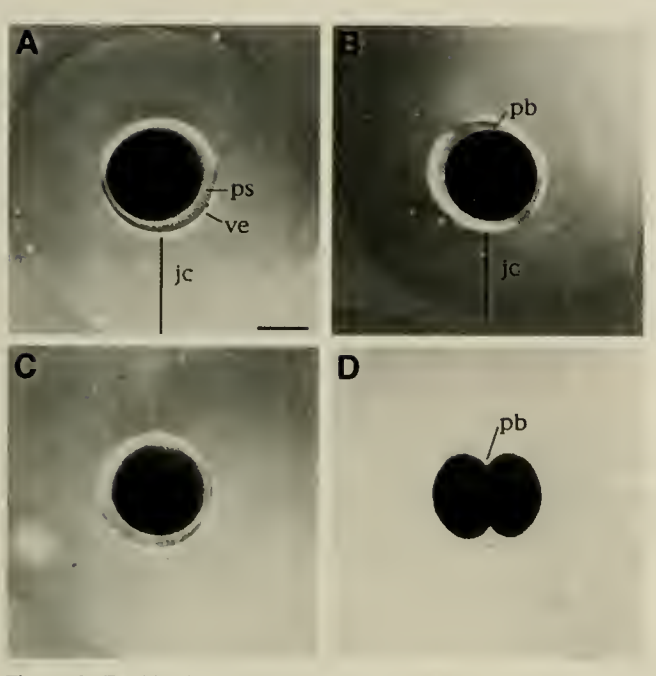

Figure 3. Fertilization of spawned eggs at 24 °C. All photomicrographs are whole mounts of living eggs, zygotes or embryos. The animal pole is towards the top in all figures. A) Spawned mature oocyte in metaphase of the first meiotic division. A thin vitelline envelope (ve; less than 1 µm thick) surrounds the light green oocyte; these are separated by a perivitelline space (ps) of about 20 µm. A jelly coat (jc) layer surrounds the egg. The egg and jelly coat together have a diameter of about 600 µm. B) Zygote approximately 15 min after fertilization. First polar body (pb) is evident and a dark green cytoplasm begins migrating towards the vegetal hemisphere. The jelly coat is still about 600 µm in diameter. C) Zygote approximately 25 min after fertilization. Cytoplasmic rearrangement continues such that the vegetal hemisphere is dark green and animal hemisphere yellow. The jelly coat is no longer detected. D) Two-cell embryo approximately 35 min after fertilization. Two polar bodies are located on the animal side of the cleavage plane. The vitelline envelope is present, but not the jelly coat. Scale bar: 100 µm.

was a jelly coat layer that was approximately 180 µm thick (Fig. 3A). Within 15 min of fertilization the first polar body was detected at the animal pole and within 25 min the jelly coat was no longer evident (Fig. 3, B and C). During this period the egg cytoplasm underwent dramatic rearrangements such that the zygote had a dark green vegetal hemisphere, yellow animal hemisphere, and a white equatorial band.

DISCUSSION

Both gonad maturity and spawning behavior in *H. asinina* on Heron Reef is seasonal, with fecund abalone spawning during the summer from October to April. Analysis of spawning during the summer reproductive season indicates that all individuals undergo synchronous spawning, with gametes released approximately every 14 d (this study, Counihan et al. in press). This predictable semilunar spawning pattern is unique amongst the haliotids. *H. asinina* populations in The Philippines and Thailand are fecund for most of the year and captive populations exhibit asynchronous spawning behavior (Singhagraiwan and Doi 1992, Jarayabhand and Paphavasit 1996, Capinpin et al. 1998). Unpredictable synchronous spawning of *H. asinina* occurs on Panagatan Reef, Antique, Philippines (Capinpin 1995) and recently captured *H. asinina* spawn more synchronously than longer-term captive abalone (Capinpin et al. 1998).

In this study we exploited the predictable spawning cycle of *H. asinina* from the southern Great Barrier Reef to obtain a detailed understanding of the timing of developmental and structural changes in abalone ovaries during the natural reproductive season. Because all individuals in the population develop in synchrony we were able to acquire a set of histological samples from individuals at different times of the spawning cycle that together reflect the timing of oogenesis and gonad development. Since in other abalone the timing of natural spawning events is less predictable and often asynchronous (Webber and Giese 1969, Shepherd and Laws 1974, Hahn 1989, Hooker and Creese 1995, Wilson and Schiel 1995), it is difficult to acquire a comprehensive developmental time course by invasive methods. The *H. asinina* reproductive cycle provides a tractable experimental system to investigate natural endogenous and exogenous factors controlling gonad development and spawning behavior in abalone. Importantly, comparison of gametogenesis and gonad organization and development in *H. asinina* with that existing in other abalone (reviewed in Hahn 1989) indicates that these processes and structures are nearly identical in tropical and temperate abalone. This suggests that insights into the regulation of reproduction and spawning in *H. asinina* are likely to be applicable to other haliotids.

Underlying macroscopic changes in ovary structure (i.e. those observed by non-invasive techniques) are stereotypic changes in trabecular connective tissue morphology and the oocytes. Ovarian trabeculae degeneration and *de novo* formation of the lumen is restricted to the area in the middle of the ovary near to the hepatopancreas and is considerably less than that observed for other species of abalone (e.g. Tutschulte and Connell 1981). The rapid reformation of trabeculae within 2 d of spawning probably results from the reduced, localized nature of the pre-spawning breakdown. Associated with the restructuring trabeculae and germinal epithelium is a cohort of early primary oocytes (termed Cohort II oocytes in this study) and cohort of larger vitellogenic primary oocytes (Cohort I oocytes). Follicle cells present at the base of Cohort I oocytes, at the connection to the trabeculae, may be contributing yolk to the developing oocytes (Dohmen 1983, de Jong-Brink et al. 1983, Hahn 1989, Voltzow 1994). These Cohort I oocytes appear to have grown close to full size within 4 d of the previous spawning (compare Figs. 1, A and B with 1, H and I). In contrast the cohort of smaller Cohort II oocytes has not increased proportionally in size during the same period (compare Figs. 1, A and B with 1, H and I; Fig. 4).

Analysis of the progression of both these oocyte cohorts through a single 2-wk spawning cycle suggests that oogenesis from a small primary oocyte (approximately 12–18 µm) to mature oocyte that will be spawned takes more than 28 d (Fig. 4). The presence of different-sized oogonia shortly after a spawning event suggests that some of these stem cells have begun oogenesis. Together, these data suggest that the entire process of oogenesis is slightly longer than 28 d and may be as long as 40 d.

The early/previtellogenic primary oocyte is actively transcribing rRNA genes, but not accumulating large amounts of yolk. The rate of yolk deposition in these oocytes begins increasing around the time of spawning of the older oocytes (i.e. about halfway through oogenesis) and vitellogenesis appears to be completed 4 to 5 d later. During the next 8 to 9 d of gametogenesis there is no obvious change in oocyte size or histospecific character; these

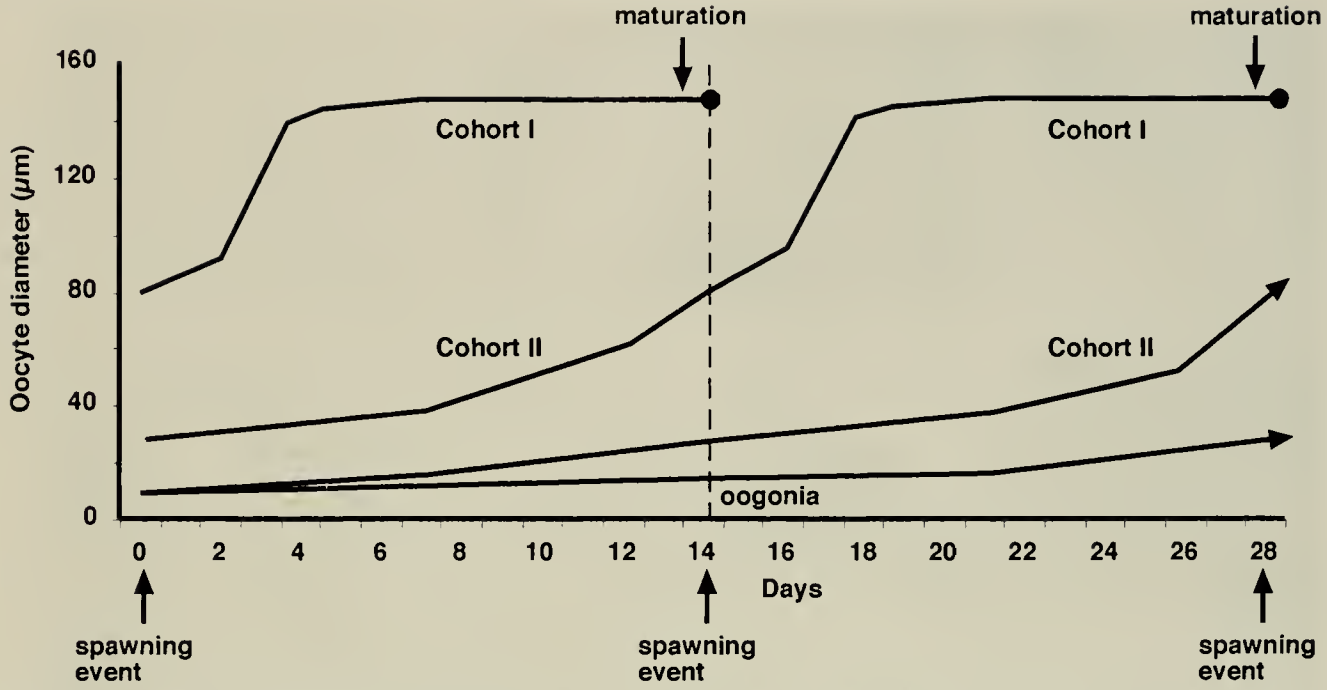

Figure 4. Oocyte growth and maturation in *H. asinina* during two consecutive spawning cycles. Maturation of Cohort 1 oocytes occurs 12 to 18 h prior to spawning. At any point in the spawning cycle the ovary contains two or three cohorts of primary oocytes and oogonial stem cells. Balls at the end of the line represent oocytes that will be spawned; arrows represent oocytes that will continue to develop and grow.

older oocytes still appear to be actively transcribing rRNA genes, as nucleoli are still present. During this period the packed polygonal-shaped oocytes begin to produce a set of extracellular layers that can be discerned by refractive properties under differential interference contrast microscopy (Figs. 1 and 2). The inner refractive layer has been called the vitelline layer (Young and DeMartini 1970, Lewis et al. 1982), while the clear outer layer has been called the chorion (Young and DeMartini 1970) or jelly coat (Lewis et al. 1982). We have used the term jelly coat, as this layer appears to be very similar to an identically named structure in sea urchin eggs. As in sea urchins the jelly coat is a transient layer associated with spawned *H. asinina* eggs, disappearing shortly after fertilization.

Like many gastropods (Dohmen 1983, de Jong-Brink et al. 1983, Longo 1983), *H. asinina* and other abalone (Hahn 1989) oocytes mature shortly before release, undergoing GVBD and remaining at metaphase of the first meiotic division until fertilization. We have observed that the onset of maturation does not begin until the oocytes are dissociated from the trabeculae, some 12 to 18 h before spawning, although we did not determine if the process of dissociation is the agent that induces the onset of maturation. Nonetheless there must exist a mechanism to ensure that only large Cohort I oocytes dissociate and not the immature Cohort II, as we did not detect the release of any small oocytes during oviduct formation. This differential release of the older oocytes also occurs in other abalone (see Tutschulte and Connell 1981).

Attempts to induce spawning artificially in *H. asinina* with a range of chemical and environmental factors that are known to be effective in inducing other abalone to spawn (Morse et al. 1977, Hahn 1989) has been problematic. Methods used for other abalone (i.e. hydrogen peroxide sea water, ultraviolet- (UV) irradiated seawater, desiccation, and thermal shock) have been shown to be ineffective for *H. asinina* (Singhagraiwan and Sasaki 1991, Singhagraiwan and Doi 1992, Jarayabhand and Paphavasit 1996, Capinpin et al. 1998, personal observations). We suggest that the protracted period between onset of maturation and spawning contributes to this lack of success. While the exact mechanisms by which these treatments induce oocyte release from the trabeculae, oocyte maturation and spawning behavior are unknown, Morse et al. (1977) suggested that hydrogen peroxide and UV light activate prostaglandin endoperoxide-forming cyclooxygenase which synthesizes a product that induces spawning. It has not been determined if this activation is required for oocyte maturation, spawning, or both processes, although hydrogen peroxide or UV light treatments typically result in mature eggs being spawned a few hours later; *H. rufescens*, *H. diversicolor diversicolor*, and *H. discus hannai* spawn 2.5–5, ~1, and ~1.5 h, respectively, after treatment begins (Morse et al. 1977, Takashima et al. 1978, Uki and Kikuchi 1984). In *H. asinina*, hydrogen peroxide has been shown to induce spawning behavior without the release of gametes (Capinpin 1995). Together, these data suggest that the inability of well-proven inducers of spawning to affect *H. asinina* may be because oocyte maturation and spawning are temporally uncoupled compared to other species.

We propose that ovulation and spawning in *H. asinina* requires two steps, with both being associated with the tidal cycle. Ovulation, which includes trabeculae degeneration, Cohort 1 oocyte maturation, and *de novo* formation of a lumen, begins during a morning low tide that occurs around a new or full moon (i.e. spring low tide). Once females have ovulated they are competent to spawn. Spawning occurs at the next evening high tide, approximately 18 h later. While individual female *H. asinina* are able to spawn over two consecutive evening high tides (Counihan et al. in press), we did not determine if there are two ovulation events. While both ovulation and spawning are correlated with spring tides, endogenous signals are sufficient to induce both these events. *H. asinina* removed from natural tidal stimuli for a limited

period (i.e. up to about 6 wk) still spawn viable gametes in synchrony with recently captured individuals (Counihan et al. in press). These endogenous rhythms appear to be set and maintained by lunar and tidal cycles.

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POPULATION GENETIC ANALYSIS OF THE ABALONE *HALIOTIS FULGENS* (MOLLUSCA: GASTROPODA) IN BAJA CALIFORNIA, MÉXICO

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ABSTRACT Population genetic structure was analyzed for *Haliotis fulgens* in the central part of Baja California, México. The study was carried out based on five abalone sites using electrophoresis analysis. Allozyme data for seven loci from individuals within abalone sites were obtained, and *F* statistics were used to assess population structure. Allozyme variation was low, but more similar than the one reported in other haliotids. Malate dehydrogenase (MDH-1 and MDH-2) and glutamate oxalate transaminase (GOT) in some population were fixed. There were not observed significant deviations from Hardy-Weinberg equilibrium among 31 comparisons made over all loci and all populations. *F* statistics did not indicate departure from random mating or evidence of genetic structuring of the *H. fulgens* populations, however F_{IT} and F_{IS} data showed that inbreeding occurs inside abalone sites. Additionally, cluster analysis and Mantel tests showed that genetic differences among abalone populations were not accumulative geographically. Data do not suggest that *H. fulgens* populations are genetically isolated from one another. The five populations formed a homogeneous group respect to allele frequencies at six of the seven loci analyzed. Therefore, they cannot be considered as different stocks. The results suggest that gene flow along the distribution of abalone banks could be variable depending of the coastal topography, dominant swells, and driving winds, which determine the local coastal hydrodynamic and consequently the level of larval interchange among populations.

KEY WORDS: Allozymes, *Haliotis fulgens*, gene flow, gene variation, population structure, stock, Baja California

INTRODUCTION

The abalone (*Haliotis* spp.) is one of the highest value commercial fishery resources worldwide. Haliotids are found in the mild and cold waters of the Western Pacific, Indopacific, Eastern Atlantic, and Californian Current. In Mexico they are found along the Pacific coast of the Baja California Peninsula where they are commercially exploited. Seven species exist in this area and *Haliotis fulgens* Philippi constitutes 85% of the total catch. This species is found between Santa Barbara, California and Isla Margarita, Baja California, Mexico and is most abundant along the central part of the peninsula between Isla Cedros and Punta Asunción (Guzmán del Próo 1992).

Over the last two decades the total catch of worldwide abalone fisheries has decreased (Shepherd and Brown 1993). The catch in Mexico fell 5-fold between 1970 and 1985 and for *H. fulgens* the catch decreased 3-fold during this period of time (Shepherd et al. 1991). This situation has been attributed mainly to overexploitation (Guzmán del Próo 1992).

Aquaculture, life history, and population dynamics studies have been carried out in Mexico to promote the recovery and sustainable yield of abalone fishery, however, no genetics studies have been carried out.

Awareness of the importance of genetic studies for a rational fisheries management has grown in recent years because an inadequate knowledge of genetic diversity and population structure may impede any conservation or management program.

In this context, stock identification is a crucial aspect because of the likelihood that a natural population may be composed of spatially or temporally isolated stocks as a function of topography, life history, behavior, larval dispersal, and historical circumstances (Spangler et al. 1981). Thus in the abalone fishery a basic question prevails: Do the abalone sites in each fishery region constitute the same unit population or are they independent isolated units?

Allozyme analysis may be a good method to determine genetic structure within and among site variation for a region. In haliotids this kind of study has only been carried out in *H. rubra* Leach (Brown 1991, Brown and Murray 1992) and *H. laevigata* Donovan (Brown and Murray 1992). The results show small genetic differences between populations of *H. rubra* that accumulate with geographical distance, but suggests, due to a small-scale genetic heterogeneity, that local populations are predominantly recruited from local stock. On the other hand, *H. laevigata* populations are genetically different from each other even over small distances.

In this paper we describe the pattern of population genetic structure in some *H. fulgens* sites from the Central Zone of Baja California, México using allozyme analysis. Specifically, we were interested in whether these abalone sites could be considered the same population unit or smaller independent units.

MATERIALS AND METHODS

Study Area

This study was carried out in five sites along the Central Zone of Baja California, México. Three of them were located inside Bahía Tortugas and relatively close to each other (2–5 km between them). The other two sites were located in Bahia Vizcaino, at Punta Eugenia and Malarrimo, toward the northern end of Bahía Tortugas, separated from each other by 54 km, and 30 to 84 km, respectively, from Bahia Tortugas (Figure 1).

The characteristics of each site are as follows: Los Morros (27° 39′ 14″ N, 114° 52′ 26″ W) is at the southeastern end of Bahía Tortugas. The bottom is rocky, rich in macroalgae, and mainly covered by giant kelp beds (*Macrocystis pyrifera*). This site is directly exposed to the waves and regularly exploited by fisherman because of its high population density.

La Cantina (27° 40′ 00″ N, 114° 53′ 55″ W) is in the northwestern portion of Bahía Tortugas. It features large boulders and sandy clearings, and the main vegetation is articulated coralline

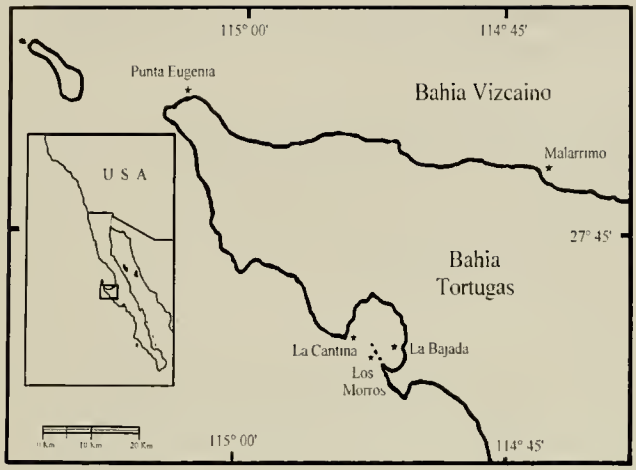

Figure. 1. Locations of the five sites sampled from Baja California Peninsula.

algae mixed with scarce giant kelp plants. This reef is partially sheltered from breaking waves. At this site, abalone density is low and commercial catch is rare.

On the southeastern side of Bahía Tortugas is La Bajada (27° 39′ 30″ N, 114° 50′ 49″ W). The sandy-stony bottom has poor vegetation and giant kelp is absent. This is a site sheltered from the breaking waves. Abalone is quite scarce and there is not a commercial catch. Punta Eugenia (27° 50′ 39″ N, 115° 04′ 35″ W) is located at the edge of Vizcaíno Peninsula. It is quite similar to Los Morros, with a stony-rocky bottom densely inhabited by *M. pyrifera*. Commercial abalone catch is important here.

Malarrimo (27° 47′ 21″ N, 114° 43′ 10″ W) is in the middle of Bahía Vizcaíno. It has a sandy-stony bottom with beds of *Phyllospadix*, coralline algae, and other red algae. Laminarial algae are scarce. The commercial catch is moderate.

Collection and Electrophoresis

Total sample size was 102 adult organisms. A higher sample size was not possible due to low commercial catch and the high market price for each specimen. The population sample per site varied from 18 to 22 organisms. Muscular tissue samples of each organism were put in a buffer solution of 0.1 M Tris-HCl, pH 7.0, and were immediately frozen in liquid nitrogen.

We used the starch-gel electrophoresis technique; enzyme stains were modified from González de León (1986). Fragments of tissue were homogenized at 4 °C, in a buffer solution of 0.1 M Tris-HCl, pH 7.0 with a Glas-Col stirrer at 2,000 rpm for 7 min. Each sample extract was stored at −70 °C until electrophoresis was conducted.

A total of 12 enzymes were assayed, but because separation and interpretation of the electromorph banding for all 12 enzymes was not clear, we decided to analyze five enzymes which offered clearly resolved bands. All migrated anodally. They were glutamate oxalate transaminase (GOT, 2.6.1.1), malic enzyme (ME, 1.1.1.40), malate dehydrogenase (MDH, 1.1.1.37), lactate dehydrogenase (LDH, 1.1.1.27), and leucine aminopeptidase (LAP, 3.4.11.1). The electrophoresis was concluded when the migration of the internal markers reached 7 to 8 cm from its origin.

Statistical Analysis

The electrophoretic data were analyzed using BIOSYS-1 (Swofford and Selander 1989) and NTSYS-PC (Rohlf 1989). The allele frequencies and the basic genetic parameters of heterozygosity, polymorphism, and number of alleles per locus were calculated for each population. Due to the difficulties encountered in using chi-square distribution for small samples, we used the exact probability test (Haldane 1954, Elston and Forthofer 1977, Weir 1996) to test conformance of allele frequencies to Hardy-Weinberg equilibrium ratio. In addition, we used the sequential Bonferroni test to reduce the tablewide type-I error rate (Rice 1989). The magnitude and direction of departure from expectations were quantified by the fixation index (F_{IS}). High levels of selfing would be reflected in high F_{IS} positive values, indicating heterozygous deficiencies.

The geographic heterogeneity of allele frequencies at each locus was tested using jackknifed standard errors of the locus-specific F_{ST} estimates, jackknifing across the five populations (*sensu* Weir and Cockerham 1984). Then, a jackknifed standard error for the overall F_{ST} estimate was determined by jackknifing across loci. Finally, we performed one-tailed t tests to test the hypothesis that $F_{ST} > 0$.

The genetic population structure was analyzed by means of F_{IT}, F_{IS}, and F_{ST} statistics (Wright 1951, Wright 1978) to describe non-random mating within and among populations. F_{IT} is the total reduction in heterozygosity of an individual due to the effects of non-random mating and population subdivision combined. It may be hierarchically partitioned to describe deviations from Hardy-Weinberg expectations on different geographic scales. The partitioning is $(1 - F_{IT}) = (1 - F_{IS})\ (1 - F_{ST})$, where F_{IS} is the reduction in heterozygosity of an individual relative to the subpopulation due to non-random mating and F_{ST} is the total reduction in heterozygosity of an individual due to population subdivision. Also, F_{ST} is a common statistic for describing average differentiation among populations.

F statistics have been derived from three perspectives: as the degree to which alleles, identical by descent, are distributed within and among individuals and populations (Malécot 1969; Nei 1973); as correlations between alleles in uniting gametes within and among subpopulations (Wright 1978); and from a nested ANOVA model where total allelic variation is partitioned among subpopulations, among individuals within subpopulations, and between pairs of alleles within individuals (Cockerham 1969, Cockerham 1973, Weir and Cockerham 1984). Although all of them yield the same result and their interpretations are biologically equivalent, we used Weir and Cockerham's F statistics because their method explicitly takes into account differences in sample sizes among the populations tested. For the significance of F statistical, we report the standard errors of the mean estimate based on the jackknife procedure across loci, and the 99% confidence limits based on the bootstrap analysis across loci (Weir 1996).

Levels of gene flow among populations were inferred from F_{ST}. This estimator is related to the number of migrants per generation (N_em), the historical average number of individuals exchanged per generation between populations, by the equation $F_{ST} \sim 1 / (4\ N_em + 1)$ (Wright 1951). However, we calculated gene flow using the relation $F_{ST} \sim 1 / (4\ N_em\ (n/n - 1)^2 + 1)$ because it takes into account the number of sampled populations (n) (Takahata 1983, Takahata and Nei 1984, Chakraborty and Leimar 1987). Although the relationship between F_{ST} and N_em is based on an infinite island model, the last equation provides a relatively robust estimate of gene flow for populations with other population genetic structure models (Crow and Aoki 1984, Slatkin and Barton 1989).

The *D* statistic (Nei 1972) was used to estimate the genetic distance among populations. A phenogram based on Nei coefficients using UPGMA was generated. The branching points standard error was estimated by means of genetic distance data and their variances. The clustering significance among populations was accepted when the standard error bar at the branching point was less than one-half of the branch length (Nei et al. 1985).

To find possible patterns of spatial differentiation, we have compared the genetic and geographic distance matrices by Mantel's non-parametric test (Manly 1997). When the correlation between matrices of geographic distance and genetic distance was significantly greater than the correlation between the geographic distance matrix and randomized matrices of genetic distance, the relationship was accepted as a significant one. Shuffling rows and columns of the original matrix of genetic distance generated randomized matrices. We used 5,000 iterations to build the randomization distribution to reach 95% confidence intervals of the correlation matrix.

RESULTS

Of the five enzymatic systems studied, seven loci were found: MDH-1, MDH-2, LDH, ME, LAP-1, LAP-2, and GOT. Banding patterns at each of them were consistent with Mendelian segregation. The allele frequencies at each locus were dominated by the same allele in all sites and showed little variability between them (Table 1).

Heterozygosity at loci varied widely within and among populations (Table 2). The mean direct count heterozygosity per population ranged from 0.054 to 0.195, the mean number of alleles per locus ranged from 1.7 to 2.0, and the percentage of polymorphic loci was highly variable among samples ranging from 14% to 100%. The lowest values both of mean heterozygosity and polymorphism were found in La Bajada and the highest were found in La Cantina (Table 2). The genetic diversity did not show a reduction or increase pattern consistent with geographic distance among populations, or in moving from south to north.

Observed mean heterozygosities differed significantly from those expected under conditions of Hardy-Weinberg equilibrium (Table 1). Using the exact test we found six significant deviations in gene frequencies of 31 comparisons made over all loci, all populations (Table 3), and all heterozygous deficits. However, the sequential Bonferroni adjustment of the *P* values showed that these deviations Hardy-Weinberg equilibrium were the result of chance alone.

The allele frequency variation across populations produced heterogeneous estimates of F_{ST} ranging from 0.0064 to 0.1355. However, each of the locus-specific estimates of F_{ST}, except MDH-1, was not significantly different than zero as revealed by *t* test utilizing the jackknifed errors (Table 4).

F statistics did not indicate departure from random mating in the *H. fulgens* populations (Table 5). F_{IS} was large and highly variable by more than a factor of 7 across loci, but only MDH-1 was statistically different from zero ($P < 0.01$). F_{IT} was less variable and differed by no more than a factor of 3 across loci, only MDH-1, ME, and LAP-2 were statistically different from zero ($P < 0.01$). Finally, F_{ST} across loci and overall populations was small and not significantly different from zero ($P < 0.01$), except MDH-1.

The average N_em, among all populations was 4.3. Gene flow between population pairs was highly variable and ranged from 0.066 to infinity (Table 6). Low gene flow ($N_em < 1$) was observed in Bahía Tortugas between La Bajada and Los Morros, and between La Bajada and La Cantina; however, Los Morros and La

TABLE 1.

Allele frequencies at seven polimorphic loci in five populations of *H. fulgens* from Baja California, México.

		Populations				
	Allele	**La Bajada**	**Los Morros**	**La Cantina**	**Malarrimo**	**P. Eugenia**
n		22	20	22	18	20
MDH-1	A	0.952	0.975	0.818	1.000	1.000
	B	0.048	0.025	0.182	0.000	0.000
	H_L	0.095	0.050	0.273		
MDH-2	A	1.000	0.800	0.795	0.944	0.895
	B	0.000	0.200	0.205	0.056	0.105
	H_L		0.300	0.227	0.111	0.105
LDH	A	0.810	0.875	0.932	0.750	0.895
	B	0.190	0.125	0.068	0.250	0.105
	H_L	0.190	0.050	0.136	0.167	0.211
ME	A	0.976	0.975	0.886	0.972	0.947
	B	0.024	0.025	0.114	0.028	0.053
	H_L	0.048	0.050	0.136	0.056	0.105
LAP-1	A	0.976	0.850	0.705	0.917	0.842
	B	0.024	0.150	0.295	0.083	0.158
	H_L	0.048	0.100	0.409	0.167	0.105
LAP-2	A	0.952	0.800	0.795	0.944	0.895
	B	0.048	0.200	0.205	0.056	0.105
	H_L		0.200	0.046	0.111	0.211
GOT	A	1.000	0.900	0.841	0.972	0.974
	B	0.000	0.100	0.159	0.028	0.026
	H_L		0.200	0.136	0.056	0.053

TABLE 2.

Summary statistics (means and standard errors in parentheses where appropriate) describing genetic variation in five populations of *H. fulgens* from Baja California, México. A locus was considered polymorphic if the frequency of the common allele was 0.95 or less.

Population	Mean Sample Size Per Locus	Mean No. of Alleles Per Locus	Percentaje of Loci Polymorphic	Mean Heterozygosity	
				Direct Count	Hardy-Weinberg Expected
La Bajada	21.0	1.7	14.3	0.054	0.085
	(0.0)	(0.2)		(0.026)	(0.041)
Los Morros	20.0	2.0	71.4	0.136	0.204
	(0.)	(0.0)		(0.037)	(0.044)
La Cantina	22.0	2.0	100	0.195	0.287
	(0.0)	(0.0)		(0.045)	(0.036)
Malarrimo	18.0	1.9	57.1	0.095	0.124
	(0.0)	(0.1)		(0.023)	(0.024)
P. Eugenia	19.0	1.9	71.4	0.113	0.144
	(0.0)	(0.1)		(0.29)	(0.036)

Cantina showed high gene flow ($N_em > 1$). Malarrimo and Punta Eugenia, separated by nearly 40 km, showed relatively high levels of gene flow (N_em 1). The gene flow between these populations and Bahía Tortugas populations was variable ($0.066 < N_em <$ infinity) and no association between levels of gene flow and geographic distance was found.

Mean genetic distance among populations was low (D_{NEI} = 0.0122, σ = 0.009; Table 7). The phenogram developed to estimate relationships among the five *H. fulgens* populations (cophenetic correlation = 0.795) yielded three clusters (Figure 2). The first formed by La Bajada and Malarrimo at a distance of 0.0019. The second formed by Los Morros and Punta Eugenia (D_{NEI} = 0.0037), which is linked to the first cluster at a distance of 0.01. At the base of the phenogram, La Cantina is associated to both clusters at a distance of 0.0201.

Nei's *D* values derived from the paired comparison of individual populations were not correlated with the geographical distance of the populations (Mantel test, r = 0.0935, t = 0.2346, P = 0.5927; Figure 3). The scatter plot did not show an island or stepping stone model among populations.

DISCUSSION

Heterozygosity and the genetic distance are influenced by the number individuals and the number of loci examined (Nei and Roychoudhury 1974, Nei 1978). Likewise, particularly the heterozygosity has been negatively correlated with the number of loci investigated (Hartl et al. 1994). For these reasons, at a first examination of our data we thought that the average heterozygosity of *H. fulgens* could has been overestimated because we studied only seven polymorphic loci. However, in spite of the low number of loci examined, the average heterozygosity we found among *H. fulgens* populations (H_A = 0.119) was similar to those reported for *H. discus hannai* Ino (H_A = 0.123; Fujio et al. 1983), *H. laevigata* (H_A = 0.195), *H. rubra* (H_A = 0.140; Brown and Murray 1992), and other mollusk (H_A = 0.129; Fujio et al. 1983). In addition, our estimates of heterozygosity were also lower than those previously reported for other benthic marine organisms (H_A = 0.285; Nevo 1978, Nevo et al. 1984), where a minimum of 15 loci were analyzed.

Another potentially problematic aspect of our study was the sample size. Several authors claim that large sample must be analyzed to estimate adequate patterns of genetic diversification (Lewontin 1974, Nei et al. 1983); however, Archie et al. (1989) recommend that at least 20 individuals should be studied when it is not possible to collect large samples, or particularly, for studying rare or endangered species. We were limited to analyzing 20 individuals per population because the Mexican abalone is a collapsed fishery, thus each abalone piece has a very high commercial value and therefore it is very difficult to get large samples.

It has also been shown that the number of individuals to be examined depends on the level of heterozygosity found (Nei 1978). That is to say, the sample size must be increased when heterozygosity is high. This was not our case because the levels of heterozygosity found for *H. fulgens* populations were low (<0.2).

Deviation from Hardy-Weinberg Equilibrium

While we observed fixed alleles in some loci and an apparent heterozygosity deficit in the studied populations, the sequential

TABLE 3.

Probabilities that the observed gene frequencies in five populations of *H. fulgens* from Baja California, México conform to those expected under Hardy-Weinberg equilibrium, using the exact test (Weir 1996). Significant values are given in bold.

	MDH-1	MDH-2	LDH	ME	LAP-1	LAP-2	GOT
La Bajada	1.000	–	0.172	1.000	1.000	**0.024**	–
Los Morros	1.000	1.000	**0.021**	1.000	**0.030**	0.128	1.000
La Cantina	0.536	0.177	1.000	0.223	1.000	**0.003**	0.056
Malarrimo	–	1.000	**0.031**	1.000	1.000	1.000	1.000
P. Eugenia	–	0.159	1.000	1.000	**0.036**	1.000	1.000

$P < 0.05$.

TABLE 4.

Locus-specific and overall F_{ST} values across five populations of *H. fulgens* from Baja California, México. Significance of variation among populations was determined by one tailed *t* test of independence.

Locus	F_{ST}	SE	*P*
MDH-1	0.1355	0.0812	*** s
MDH-2	0.0434	0.0440	** ns
LDH	0.0064	0.0233	** ns
ME	0.0116	0.0229	** ns
LAP-1	0.0663	0.0669	** ns
LAP-2	0.0145	0.0154	** ns
GOT	0.0450	0.0325	** ns
Overall	0.0461	0.0408	** ns

Significance level for *t* test ** $P < 0.05$, *** $P < 0.01$.

Bonferroni test suggested that none of the deviations from Hardy-Weinberg equilibrium had biological significance. This result is different from other abalone species, such as *H. discus hannai* (Fujio et al. 1983) and *H. rubra* (Brown and Murray 1992), and other bivalves such as *Mytilus edulis* L. (Kohen et al. 1976, Johannenson et al. 1990), *Crassostrea virginica* Gmelin (Singh and Zouros 1978, Buroker 1983), and *C. gigas* Thunberg (Gosling 1981) that have shown departures from Hardy-Weinberg equilibrium by heterozygous deficiency.

Although F_{IS} and F_{IT} showed a positive sign that confirmed a deficit of heterozygosity within populations, they were not statistically different from zero in all the loci of *H. fulgens*. Brown and Murray (1992) found a similar pattern of heterozygous deficiency in *H. rubra* and *H. laevigata* with a positive overall for both F_{IS} and F_{IT}, but the heterozygous deficiency were not consistent across all loci.

The estimated F_{ST} across populations surveyed do not suggest that *H. fulgens* populations are genetically isolated from one another. In fact only 4% observed genetic variability is due to geographic subdivision and random genetic drift. Furthermore, the five populations studied formed a homogeneous group with respect to allele frequencies at six of the seven loci analyzed (Table 4). Therefore, we cannot consider these populations as different stocks.

TABLE 5.

Weir and Cockerham (1984) estimates of Wright's *F* values calculated separately for each locus for population of *H. fulgens* from Baja California, México. Means and standard errors were obtained by jackknifing over loci. Confidence interval obtained by bootstrapping over loci.

Locus	F_{IS}	F_{IT}	F_{ST}
MDH-1	0.0707	0.1540	0.0897
MDH-2	0.2360	0.2750	0.0510
LDH	0.3962	0.4000	0.0063
ME	0.1602	0.1634	0.0038
LAP-1	0.2858	0.3264	0.0568
LAP-2	0.4941	0.5025	0.0166
GOT	0.2371	0.2704	0.0437
Mean (SD)	0.3180 (0.051)	0.3351 (0.044)	0.0358 (0.010)
99% Confidence Interval	0.1289–0.5070	0.1868–0.4833	0.0–0.0728

TABLE 6.

Number of migrants per generation for five populations of *H. fulgens* from Baja California, México. Pairwise estimates of gene flow (N_em) based on Weir and Cockerham's (1984) F_{ST}.

	Los Morros	La Cantina	Malarrimo	P. Eugenia
La Bajada	0.932	0.439	infinity	2.720
Los Morros		15.182	2.135	infinity
La Cantina			0.066	0.153
Malarrimo				10.435

Genetic Differentiation

The small genetic distance observed among populations ($0.001 \leq D \leq 0.029$) confirms that they are genetically homogeneous and form part of an interconnected large population by gene flow. Nevertheless, the phenogram and Mantel's test showed that the differences among populations were small and not accumulative geographically because none of them was joined to another by its geographic proximity (Figures 2 and 3). A different pattern was described by Brown and Murray (1992) for *H. rubra*, where a broad-scale gene pool for this species was geographically homogeneous and accumulative.

Although all the analysis leads us to conclude that the five populations surveyed from part of a large population, two important issues that emerged from this study remain to be explained: (1) why we observed a high degree of homozygocity in *H. fulgens* populations, despite the fact that F_{IS} and F_{IT} do not differ significantly from zero, and (2) how can we explain the heterogeneous flow observed?

In the first case, we hypothesize that inbreeding and genetic drift could be provoking the heterozygous deficiency observed within populations of *H. fulgens* in Baja California, although selection against heterozygous could be another explanation. In fact the inbreeding and genetic drive could be caused by a combination of factors, such as the reduction of the adult population size observed in the last 20 years as a result of a serial over-exploitation of small population units within the larger fishery that the Mexican abalone was in early times (Prince and Guzmán del Próo 1993), and the limited larval dispersal characteristic of these species to a scale of tens to hundreds of meters (Prince et al. 1987, Prince et al. 1988) up to several kilometers (McShane et al. 1988, Shepherd and Brown 1993, Sasaki and Shepherd 1995).

Particularly, recent hydrodynamic experiments in Bahia Tortugas have shown that the average currents could potentially transport larvae for 3 to 5 km along shore during the first 5 days of the

TABLE 7.

Nei's genetic distance (Nei 1972) above the diagonal and the geographical distances (kilometers) below the diagonal among five populations of *H. fulgens* from Baja California, México.

	La Bajada	Los Morros	La Cantina	Malarrimo	P. Eugenia
La Bajada		0.0135	0.0294	0.0019	0.0065
Los Morros	2.7		0.0100	0.0105	0.0038
La Cantina	5.2	2.8		0.0277	0.0133
Malarrimo	68.8	66.4	63.6		0.0052
P. Eugenia	32.4	30.0	37.2	36.4	

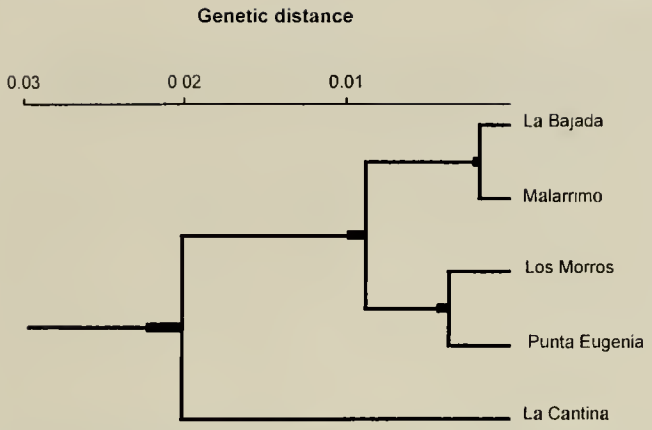

Figure. 2. Phenogram showing the genetic relationships of five *H. fulgens* localities from Baja California Peninsula. Nei's genetic distance were clustered using UPGMA. The bars represent the standard error. Cophenetic correlation was 0.799.

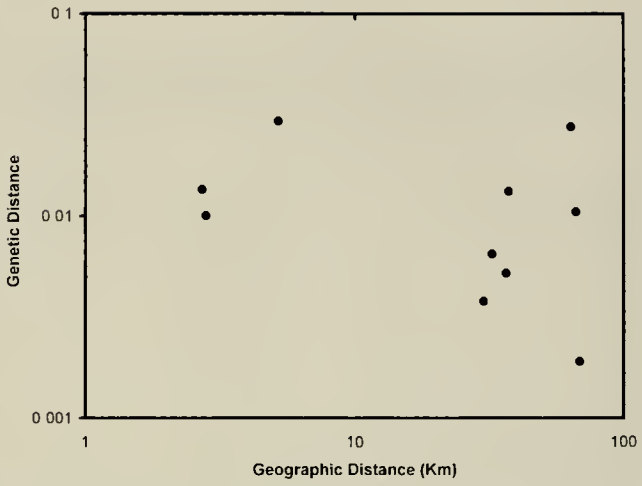

Figure. 3. Scatter plots demonstrating the no-relationship between genetic distance and geographic distance.

pelagic cycle. (Guzmán del Próo et al. in press). Thus a limited larval dispersal in the neighborhood of the breeding population increases the larvae survival, however, it also increases the inbreeding and local competition within populations due to a reduced gene flow.

Regarding the heterogeneous gene flow observed among five populations of *H. fulgens* analyzed, we also could explain it because of the limited larval dispersal. The hydrodynamic pattern in Bahía Tortugas and neighboring coastal zone showed that larval exchange could potentially take place between neighboring abalone reefs at a limited distance under dominant swells coming from the west. Nevertheless, it does not exclude the possibility of some reduced interchange of larvae between more distant reefs, providing the dominant hydrodynamic conditions change, for instance, when the direction of swells coming from the west change southerly, the intensity of the winds changes, or the tidal low changes (Guzmán del Próo et al. in press). Thus we hypothesize that gene flow along the distribution of abalone reefs could be variable depending of the coastal topography, dominant swells, and driving winds that determine the local coastal hydrodynamic and consequently the level of larval interchange among reefs.

These types of findings have important implications in terms of fishery management. Modern fisheries science demands a holistic management of the fisheries; this concept includes the maintenance of genetic diversity and the population structure, which are critical for ensuring the long-term survival of any fishery (Shepherd and Brown 1993). Although this study was confined to a limited area, the results are likely to be indicative of population genetics of Mexican abalone and the factors influencing it, which should be considered in future management policy.

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IRON METHIONINE (FeMET) AND IRON SULFATE ($FeSO_4$) AS SOURCES OF DIETARY IRON FOR JUVENILE ABALONE, *HALIOTIS DISCUS HANNAI* INO.

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ABSTRACT The minimum dietary iron requirement and its bioavailability were determined for juvenile abalone (*Haliotis discus hannai* Ino.) using casein-gelatin-based diets supplemented with 0, 10, 20, 30, 60, 120, and 200 mg iron/kg from iron methionine (FeMet) or iron sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$). The experimental diets containing graded levels of dietary iron (24.9–212.7 mg iron/kg) provided as either FeMet or $FeSO_4$ were fed to juvenile abalone. Abalone juveniles of similar size were distributed in a flow-through system using a completely randomized design with thirteen treatments and three replicates of each treatment. Abalone fed the basal diet without iron supplementation exhibited significantly lower survival and carcass protein than the other groups. The average weight gain rate (WGR, %), daily increment in shell length (DISL, μm/day), and soft-body iron concentration (SB iron, μg/g) of the abalone were significantly affected by dietary treatment, and responded in broken-line models to increases in dietary iron levels from the two iron sources. The optimal levels of dietary iron using FeMet and $FeSO_4$ as the supplemental iron sources, determined by broken-line regression analysis, on the basis of maximum WGR, were 62.79 and 58.35 mg/kg, respectively. On maximum DISL, the values were 66.12 and 62.11 mg/kg, respectively, and on maximum SB iron deposition were 66.78 and 64.55 mg/kg, respectively. The content of iron and manganese in the shell, however, was maintained relatively constant regardless of dietary treatment. Significantly reduced manganese deposition in soft-body parts was observed for the abalone fed diets containing high levels (≥100 mg/kg) of iron from $FeSO_4$. However, this effect of excess iron on the utilization of manganese was not found when using FeMet as the iron source. Based on these results, an optimal level of dietary iron was recommended to be 65–70 mg/kg with either FeMet or $FeSO_4$ as the iron source. This experiment also showed that the bioavailability of dietary iron from $FeSO_4$ was as high as that from FeMet.

KEY WORDS: abalone, *Haliotis discus hannai*, iron requirement, bioavailability, nutrition, mollusks

INTRODUCTION

Iron is an essential trace element in all-higher animals, including fish, because of its importance in cellular respiration and mitosis (Robbins et al. 1972). Studies on dietary iron have been conducted with some fish species. Iron deficiency in channel catfish, *Ictalurus punctatus*, has been characterized by suppressed hematocrit, hemoglobin, plasma iron content and transferrin saturation, and a dietary iron requirement of ≤30 mg/kg diet was recommended for this species (Gatlin and Wilson 1986). A requirement of 150 mg/kg diet has been reported for red sea bream, *Chrysophrus major*, (Sakamoto and Yone 1978a). All these recommended values were evaluated when iron sulfate ($FeSO_4 \cdot 7H_2O$) was the source of supplemental iron.

The nutritional value of dietary mineral sources depends not only upon their contents in the feedstuff but also upon the bioavailability of the element to animals (Paripatananont and Lovell 1997). Studies with mammals have shown that chelation of minerals to amino acids may increase their absorption rate in the intestine (Ashmead 1992). Wedekind et al. (1992) reported that the bioavailability of zinc from zinc methionine (ZnMet) in poultry was greater than that from zinc sulfate ($ZnSO_4$). Paripananont and Lovell (1995) also reported that using ZnMet as the dietary zinc source reduced the optimal levels of dietary zinc for channel catfish in both purified and practical diets as compared to the diets containing $ZnSO_4$. More recently, Paripananont and Lovell (1997) reported that the coefficients of net absorption of chelated trace minerals (Zn, Fe, Cu, Mn, and Se) were much higher than those of inorganic forms of these minerals for channel catfish in both purified and practical diets. Similar results were observed by Li et al. (1995) in shrimp (*Penaeus chinensis*) in a practical diet. However, Lim et al. (1996) reported that iron methionine and iron sulfate were equally effective in improving growth and preventing anemia in channel catfish.

Excess iron supplementation may, however, be detrimental because of the pro-oxidant nature of this mineral (Gatlin and Wilson 1986) and decreasing absorption of other minerals such as manganese (Johnson and Korynta 1992). In addition, unnecessarily high additions of iron and other micronutrients also increase the price of feeds, as well as increase the input of minerals to the aquatic environment.

There is no information on the requirement of dietary iron in any mollusc species, including juvenile abalone, *Haliotis discus hannai* Ino., one of the most widely cultured and commercially important abalone. Also, no information is available on the bioavailabilities of organic and inorganic sources of dietary iron to mollusc species. Therefore, the objectives of this study were to determine the dietary iron requirement of juvenile abalone, *H. discus hannai*, with FeMet and $FeSO_4$ as the iron sources and to compare the bioavailabilities of the two iron sources using a premium quality diet, based on casein-gelatin as the protein sources. Determination of the iron requirement and bioavailabilities of this species was not only based on the growth and survival of the animal, but also on the tissue iron deposition.

MATERIALS AND METHODS

Feed Formulation and Manufacture

The basal diet formulation is given in Table 1. Casein and gelatin were used as protein sources. Crude protein level of the experimental diets was about 30%, which is considered to be sufficient to maintain optimum growth for *H. discus hannai* (Mai et al. 1995b). Soybean oil and menhaden fish oil (1:1) was used as the basal lipid source. Dietary lipid level was about 3.5%, which

TABLE 1.

Composition of the basal diet (dry weight basis).

	Percent in Diet
Ingredient	
Casein, vitamin-free (Sigma Chemical, St. Louis, MO)	25.00
Gelatin (Sigma Chemical, St. Louis, MO)	6.00
Dextrin (Shanghai Chemical Co., Shanghai, China)	38.50
Sodium alginate (Shanghai Chemical Co., Shanghai, China)	20.00
SO/MFO (Food grade)[a]	3.50
Choline chloride (Shanghai Chemical Co., Shanghai, China)	.50
Fe-free mineral mix[b]	4.50
Vitamin mix[c]	2.00
Proximate analysis (means of triplicate)	
Crude protein (%)	28.52
Crude lipid (%)	3.53
Ash (%)	8.28
Gross energy (kJ/g)[d]	17.19
Iron (mg/kg)	24.85

[a] Soybean oil and menhaden fish oil (1:1) with 0.001% ethoxyquin.

[b] Fe-free mineral mix, each 1000 g of diet contained: NaCl, 0.4 g; $MgSO_4 \cdot 7H_2O$, 6.0 g; $NaH_2PO_4 \cdot 2H_2O$, 10.0 g; KH_2PO_4, 20.0 g; $Ca(H_2PO_4)_2 \cdot H_2O$, 8.0 g; $ZnSO_4 \cdot 7H_2O$, 141.2 mg; $MnSO_4 \cdot H_2O$, 64.8 mg; $CuSO_4 \cdot 5H_2O$, 12.4 mg; $CoCl_2 \cdot 6H_2O$, 0.4 mg; KIO_3, 1.2 mg; Na_2SeO_3, 0.4 mg.

[c] Vitamin mix, each 1000 g of diet contained: thiamin HCl, 120 mg; riboflavin, 100 mg; folic acid, 30 mg; PABA, 400 mg; pyridoxine HCl, 40 mg; niacin, 800 mg; Ca pantothenate, 200 mg; inositol, 4000 mg; ascorbic acid, 4000 mg; biotin, 12 mg; vitamin E, 450 mg; menadione, 80 mg; B_{12}, 0.18 mg; vitamin A, 100 000 IU; vitamin D, 2000 IU; ethoxyquin, 400 mg.

[d] Estimated with an XYR-1 bomb calorimeter.

was sufficient to support optimum growth and provide enough EFA for the abalone (Mai et al. 1995a). The compositions of vitamin and mineral mixtures were modified from those used by Uki et al. (1985). The casein-gelatin-based diet contained 24.85 ± 2.71 mg/kg of intrinsic iron. The basal diet was supplemented with 0, 10, 20, 30, 60, 120, and 200 mg of iron/kg from either iron methionine (FeMet) (Feed Additive Co., Ministry of Chemistry Industry of China, Jinan) or iron sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$) (Sigma, St. Louis, MO). Final iron concentrations in the experimental diets (n = 3) were found to be: 24.85 ± 2.71, 35.52 ± 1.96, 43.48 ± 2.15, 54.33 ± 2.56, 82.17 ± 3.04, 136.51 ± 3.78, and 210.13 ± 2.86 mg/kg dry diet from FeMet, and 24.85 ± 2.71, 34.13 ± 1.74, 41.59 ± 2.11, 53.28 ± 1.68, 82.46 ± 2.12, 135.39 ± 3.17, and 212.65 ± 2.65 mg/kg dry diet from $FeSO_4$ as determined by ICP-AES.

Procedures for diet preparation were modified from those described by Mai et al. (1995a, 1995b). Casein, gelatin and some minerals that were in the form of small grains were ground individually using a Pascal Mill and then passed through a mesh with 125-μm pore size. Dry ingredients were weighed on an electronic balance and thoroughly mixed. After adding water (about 120%, v/w) to the mechanically mixed ingredients containing 20% sodium alginate, a paste was made. The paste was shaped into 0.5-mm thick sheets, which were cut into 1 cm^2 flakes. The flakes were dipped into an aqueous solution of $CaCl_2$ (5%, w/v) for 1 min. By this treatment, sodium alginate was converted to an insoluble calcium alginate gel, in which the nutrients were bound (Uki and Watanabe 1992). The surplus solution was drained naturally, then the flakes were sealed in a sample bag and stored at −20 °C until use.

Animal Rearing

Juvenile abalone, *H. discus hannai*, used in this experiment were derived from a spawning in June 1998, at Mashan Fisheries Co. Shandong, China. Before the trial, shell length was measured with calipers to the nearest 0.02 mm and the animals were weighed to the nearest 0.01 g using an electronic balance.

Animals were kept in acrylic square cages (20 cm × 20 cm × 20 cm). Each rearing unit was stocked with 25 abalone juveniles. Similar size juveniles (mean weight 0.702 ± 0.02 g; mean shell length 16.110 ± 0.10 mm) were assigned to the rearing system using a completely randomized design with thirteen treatments and three replicates per treatment. The system was flow-through, with water filtered to 30 μm by primary sand filters, then to 10 μm by secondary composite sand filters. The flow rate was about 0.5 L per min per cage. Cages were kept in dim light by screening with black plastic drapes. During the experimental period, water temperature ranged from 18.2–22.0 °C, salinity 30–34, pH 7.6–7.9. Dissolved oxygen was not less than 7 mg/L, and there were negligible levels of free ammonia and nitrite (AOAC, 1995). The rearing water contained 0.35 ± 0.1 mg Fe/L as determined by ICP-AES (n = 3).

Prior to initiation of the experiment, the abalone underwent a 2-week conditioning period during which they readily adjusted to an iron-depleted casein-gelatin-based diet (Table 1) and standardized environmental conditions. The feeding trial was run for 16 wk. Abalone were hand-fed with the test diets at a rate equaling 5–10% of abalone wet weight per day, once daily at 17:00. Every morning, uneaten feed and feces were removed to maintain the water quality.

Sample Collection and Analysis

At the termination of the experiment, animals were not fed for 3 days, then all abalone were removed from the cage, weighed, measured, and counted. Then, 15 abalone from each replicate were frozen (−20 °C) for subsequent analysis. Growth was expressed as weight gain rate (WGR, %) and daily increment in shell length (DISL, μm/day). The calculation formulae were as follows:

$$WGR(\%) = [(Wt - Wi)/Wi] \times 100$$

$$DISL = [(SLt - SLi)/t] \times 1000$$

Where Wt and Wi are final and initial mean weight (g), respectively; SLt and SLi are final and initial mean shell length (mm), respectively; and t is the feeding trial period (day).

Proximate analyses to determine protein, lipid, ash, and moisture levels were conducted using conventional procedures (AOAC 1995).

Elemental analyses of shells and soft bodies of the abalone were modified from the method described by Shearer (1984). The shell samples were digested in a mixture of equal parts of hydrochloric acid (37%, ACS reagent) and nitrite acid (70%, ACS reagent) at a ratio of 1:20 (w/v). The soft body samples were digested in perchloric acid ($HClO_4$, 70%, ACS reagent) at a ratio of 1:20 (w/v). Then, the digests were appropriately diluted with Milli-Q water within the analytical capabilities of the ICP atomic

emission spectrophotometer (JY 70Plus, Jobin Yvon Co.). Elemental concentrations of the samples are expressed on a wet-weight basis as recommended by Shearer (1984).

Leaching

The leaching test of dietary iron was carried out according to the method used by Coote et al. (1996). Pre-weighed feed was put onto 100-micron mesh screens and allowed it to settle to the bottom of experimental cages without abalone. Temperature and flow rate were adjusted to match those of the experiment, the values being 20 ± 0.8 °C, and about 0.5 L per min per cage, respectively. At the end of the allotted time (0, 6, and 12 h, respectively), the remaining feed was removed from the cages and dried overnight at 60 °C in an oven. Dried feed was submitted for analysis of total iron with an ICP-atomic emission spectrophotometer.

Statistical Analysis

Data from each treatment were subjected to one-way ANOVA. When overall differences were significant at less than 5% level, Tukey test was used to compare the mean values between individual treatments. Statistical analysis was performed using STATISTICA™ package. Dietary iron requirement of juvenile abalone was determined by the broken-line regression analysis (Robbins et al. 1979, Robbins 1986). The linear segments of the regression lines, below the breakpoints, were used to compare the bioavailability of dietary iron mainly from FeMet with that mainly from $FeSO_4$ by deriving the ratio of the slopes of the lines (Forbes and Parker 1977, Paripatananont and Lovell 1995).

RESULTS

Leaching

The results of the 12-h leaching test are illustrated in Figure 1. The iron content of the diets, supplemented with the two iron sources, all declined within the whole test period. After 6 h in seawater, the remaining iron content of the diets ranged from 20.17 to 139.37 mg/kg for FeMet diet (Fig. 1A), and from 20.17 to 137.17 mg/kg for $FeSO_4$ diet (Fig. 1B). After 12 h of immersion in seawater, the dietary iron content ranged from 18.66 to 77.97 mg/kg for FeMet diet (Fig. 1A) and from 18.66 to 78.92 for $FeSO_4$ diet (Fig. 1B). There were similar leaching rates between the two iron sources. After 6 h of immersion in seawater, the leached iron accounted for approximately 20–35% of the total iron in the diets for the two sources of iron, and this value increased to approximately 25–60% after 12 h of immersion in seawater.

Survival and Growth

At the end of 16 wk, abalone fed the basal diet, without iron supplementation, had significantly (ANOVA, $P < .01$) lower survival than abalone fed diets containing supplemental iron from either FeMet or $FeSO_4$. Abalone fed iron-supplemented diets did not differ from each other in survival, regardless of iron level (Table 2, Table 3). The average WGR (%) and DISL (μm/day) of the animals were significantly affected ($P < .01$) by the varying levels of dietary iron from the two iron sources (Table 2 and Table 3). The mean weight gain rate ranged from 61.88 to 132.41% for the abalone fed diets containing graded levels of iron from FeMet (Table 2), and from 61.88 to 137.60% for $FeSO_4$ (Table 3). Daily increment in shell length ranged from 63.99 to 85.46 μm/day for FeMet (Table 2), and from 63.96 to 86.54 μm/day for $FeSO_4$

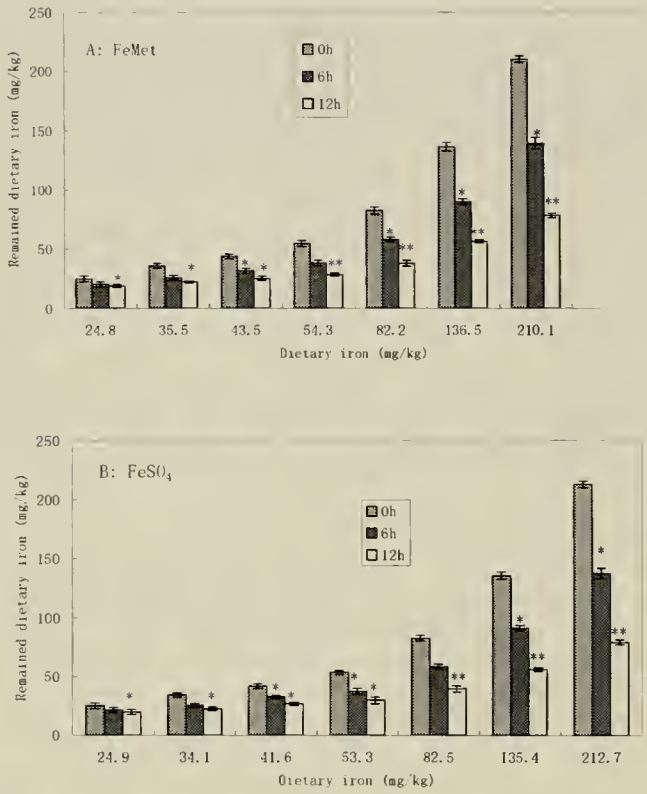

Figure 1. Changes of iron content in the diets containing varying levels of supplemental iron to the basal diet from either FeMet (A) or $FeSO_4$ (B) with increasing immersion time (0, 6, and 12 h, respectively) in seawater. Error bars are the SD, values significantly different (ANOVA, Tukey's test) from the controls (0 h) are indicated with asterisks (*$P < .05$ and **$P < .01$).

(Table 3). Both WGR and DISL responded in broken-line models to increases in dietary iron levels with the two iron sources (Fig. 2 and Fig. 3). The breakpoint in the regression line, which is considered to be the minimum dietary level for optimum response, was 62.79 mg iron/kg diet for WGR, and that was 66.12 mg iron/kg diet for DISL when using FeMet as the iron source (Fig. 2). The breakpoints for WGR and DISL were similar when using $FeSO_4$ as the iron source compared to FeMet, and the values were 58.35 and 62.11 mg iron/kg diet, respectively (Fig. 3). The ratios of the slopes of the broken-line equations were 1.09 (2.1927/2.0029) for WGR and 1.15 (0.5987/0.5196) for DISL with $FeSO_4$ as iron source compared to FeMet (Fig. 2 and Fig. 3). Thus, The bioavailability of dietary iron mainly from FeMet seems to be as high as that mainly from $FeSO_4$.

Carcass Composition

Data on percentage of soft body moisture, protein, and lipid are shown in Table 4. There were no significant differences ($P > .05$) in the contents of soft-body moisture and lipid of the abalone fed varying levels of dietary iron from the two iron sources. The content of soft-body protein, however, was significantly affected by dietary treatment. The lowest protein level was obtained in the abalone fed the basal diet, the value being 52.90%, and was effectively increased by supplementation of iron with the both sources of iron.

TABLE 2.

WGR, DISL, and survival of abalone fed graded levels of dietary iron from FeMet for 16 wk (mean ± SD, n = 3).

Added Iron (mg/kg)	Dietary Iron (mg/kg)	Initial Shell Length (mm)	Initial Weight (g)	Final Shell Length (mm)	Final Weight (g)	WGR (%)	DISL (μm/d)	Survival (%)
0	24.9	16.052 ± .14	.695 ± .02	23.088 ± .23[a]	1.124 ± .04[a]	60.88 ± 7.5[a]	63.96 ± 3.3[a]	88.00 ± 4.0[a]
10	35.5	16.111 ± .10	.704 ± .01	23.617 ± .22[a]	1.245 ± .04[b]	76.69 ± 4.3[a]	68.24 ± 1.1[a]	97.33 ± 4.6[b]
20	43.5	15.971 ± .23	.686 ± .03	24.324 ± .12[b]	1.411 ± .02[c]	105.83 ± 6.6[b]	75.93 ± 1.2[b]	98.67 ± 2.3[b]
30	54.3	16.154 ± .10	.706 ± .01	24.760 ± .10[bc]	1.527 ± .02[d]	116.38 ± 1.0[bc]	78.24 ± .3[bc]	100.00 ± .0[b]
60	82.2	16.195 ± .25	.708 ± .04	25.595 ± .17[d]	1.641 ± .03[e]	132.41 ± 11.8[c]	85.46 ± 2.4[d]	100.00 ± .0[b]
120	136.5	16.093 ± .14	.700 ± .02	25.479 ± .14[d]	1.623 ± .04[de]	131.71 ± 1.0[c]	85.33 ± .3[d]	100.00 ± .0[b]
200	210.1	16.112 ± .20	.707 ± .02	25.318 ± .46[d]	1.609 ± .06[de]	129.87 ± 5.4[c]	83.69 ± 2.6[cd]	100.00 ± .0[b]
ANOVA								
F value		.5206	.3276	57.3371	87.7834	57.9954	57.0539	9.5417
P value		.7835	.9114	.0000	.0000	.0000	.0000	.0002

[a–e] Means in the same column sharing a common superscript letter were not significantly different ($P > .05$) as determined by Tukey's test.

Elemental Concentration

The concentrations of shell ash, iron, and manganese are presented in Table 5. The contents of both the ash and the selected elements of the shells were relatively constant ($P > .05$) regardless of dietary treatment for the two iron sources.

The levels of soft-body ash, iron, and manganese are shown in Table 6. After 16 weeks of feeding trial, no significant differences ($P > .05$) were observed in the levels of soft-body ash among dietary treatments. The concentrations of iron, however, were significantly affected ($P < .01$) by the varying levels of dietary iron from the two iron sources. The iron content ranged from 312 to 528 μg/g for $FeSO_4$ and from 312 to 530 μg/g for FeMet. Iron content also responded in a broken-line model to increases in dietary iron levels with both iron sources (Fig. 4A and B). The breakpoints in the regression lines were 66.78 mg iron/kg diet for FeMet (Fig. 4A) and 64.55 mg iron/kg diet for $FeSO_4$ (Fig. 4B). The bioavailability of iron from FeMet in relative to that from $FeSO_4$ was 101% (100 × 5.2225/5.1769). The manganese content was strongly affected by the addition of iron from $FeSO_4$, while that was relatively constant irrespective of dietary iron levels from FeMet (Table 6). Namely, the manganese content of abalone fed diets containing 120 or 200 mg iron/kg was markedly lower than that of abalone fed diets containing ≤60 mg iron/kg when using $FeSO_4$ as the iron source.

DISCUSSION

The diets used in this experiment supported satisfactory abalone growth. After 16 weeks of the feeding trial, all groups that obtained sufficient dietary iron from the two iron sources grew well both in mean weight gain rate and in mean daily increment in shell length, in comparison to those reported by other authors (e.g., Uki et al. 1985, Uki and Watanabe 1992, Mai et al. 1995a, Mai et al. 1995b, Mai 1998).

To our knowledge, there is only one previously published paper pertaining to the dietary mineral nutrition of abalone, *H. laevagata*, (Coote et al. 1996). This is probably in part due to the problems associated with leaching of minerals added to the soft moist diet. In the present experiment, the special feed manufacture technology was adopted so as to improve the water stability of the feed. Leaching of dietary iron, however, still occurred (Fig. 1). We observed the fact that the digestive tracts of the most abalone were full of food within 2 h of feeding with the premium quality diets (Mai et al. 1998). This, together with the fact that a series of criteria responded in broken-line models to increases in dietary iron levels with the two iron sources, makes the requirement of dietary iron of juvenile abalone recommended in the present study acceptable. If the water stability of dietary iron can be further improved, the optimal level of dietary iron for this species may be further reduced to a certain extent.

TABLE 3.

WGR, DISL, and survival of abalone fed graded levels of dietary iron from $FeSO_4$ for 16 wk (mean ± SD, n = 3).

Added Iron (mg/kg)	Dietary Iron (mg/kg)	Initial Shell Length (mm)	Initial Weight (g)	Final Shell Length (mm)	Final Weight (g)	WGR (%)	DISL (μm/d)	Survival (%)
0	24.9	16.052 ± .14	.695 ± .02	23.088 ± .23[a]	1.124 ± .04[a]	61.88 ± 7.5[a]	63.96 ± 3.3[a]	88.00 ± 4.0[a]
10	34.1	16.149 ± .13	.709 ± .01	23.861 ± .25[b]	1.282 ± .03[b]	80.72 ± 1.6[a]	70.11 ± 1.6[ab]	100.00 ± .0[b]
20	41.6	16.191 ± .17	.713 ± .02	24.374 ± .10[b]	1.433 ± .02[c]	101.08 ± 7.5[b]	74.38 ± 1.2[b]	100.00 ± .0[b]
30	53.3	16.188 ± .10	.705 ± .01	25.106 ± .21[c]	1.575 ± .08[d]	123.25 ± 8.6[c]	81.07 ± 3.5[bc]	97.33 ± 2.3[b]
60	82.5	16.228 ± .10	.730 ± .02	25.686 ± .10[c]	1.719 ± .05[e]	135.36 ± 4.6[c]	85.15 ± 1.1[c]	97.33 ± 2.3[b]
120	135.4	15.964 ± .20	.682 ± .03	25.483 ± .13[c]	1.619 ± .06[de]	137.60 ± 5.8[c]	86.54 ± 1.6[c]	100.00 ± .0[b]
200	212.7	15.999 ± .14	.681 ± .01	25.477 ± .20[c]	1.672 ± .03[de]	137.55 ± 7.2[c]	86.17 ± 3.1[c]	100.00 ± .0[b]
ANOVA								
F value		2.2392	2.8451	60.2094	66.1033	60.1299	41.0526	15.1333
P value		.1004	.0502	.0000	.0000	.0000	.0000	.0000

[a–c] Means in the same colum sharing a common superscript letter were no significantly different ($P > .05$) as determined by Tukey's test.

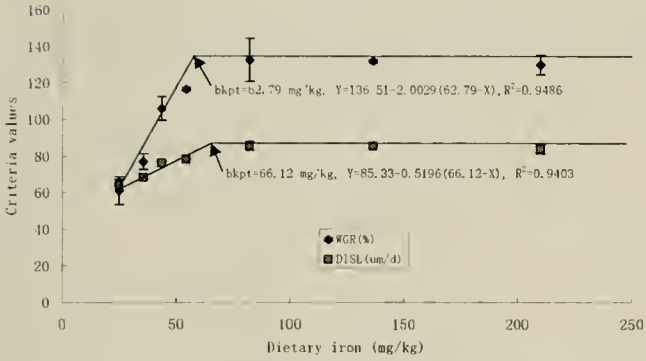

Figure 2. Regression nf WGR (%) and DISL (μm/day) on dietary iron levels and breakpoints (bkpt) in the lines for juvenile abalone fed the diets containing graded levels of iron methionine (FeMet) for 16 wk.

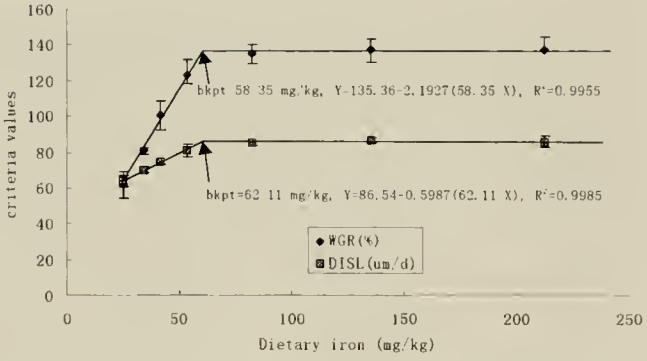

Figure 3. Regression of WGR (%) and DISL (μm/day) on dietary iron levels and breakpoints (bkpt) in the lines for juvenile abalone fed the diets containing graded levels of iron sulfate ($FeSO_4 \cdot 7H_2O$) for 16 wk.

Significantly increased mortality was observed in the abalone fed the basal diet without supplementation of iron, which contained 24.85 mg iron/kg (Tables 1 and 2). This result is similar to that reported by Sealey et al. (1997). They indicated that a deficiency of dietary iron was found to increase mortality of channel catfifh due to enteric septicemia of catfish (ESC). They also reported that the chemotactic migration by macrophages was depressed in iron-deficient fish and a level of 60 mg/kg from either FeMet or $FeSO_4$ provided the highest chemotactic indexes. The mechanisms by which iron deficiency impairs immune responses in mammalian species are not clearly understood (Sherman and Morton 1984). Iron deficiency is responsible for the reduced activity of several enzymes, including ribonuleotite reductase (Hoffbrand et al. 1976) and myeloperoxidase (Baggs and Miller 1973). Decreased protein synthesis due to the reduced activity of these enzymes may be a factor in reduced immunocompetence through decreased antibody production (Sherman and Helyar 1988). Robbins et al. (1972) reported decreased protein synthesis in mammals suffering from severe iron deficiency. In the present study, data from the carcass composition showed that the lowest protein level was obtained in the abalone fed the basal diet, and carcass protein was effectively increased by supplementation of iron from the both sources, while the content of carcass moisture and lipid was maintained relatively constant irrespective of dietary iron levels. More studies should be conducted to understand the effects of dietary iron on the immune responses and disease resistance in abalone.

Results of this study showed that WGR and DISL were the two responsive parameters to dietary iron levels from both FeMet and $FeSO_4$ and responded in broken-line models to increases in dietary iron levels with the two iron sources. Significantly depressed growth was noticed after a 16-wk feeding trial for the abalone fed low-iron diets. Impaired growth has also been observed in fishes fed low-iron diets (e.g., Gatlin and Wilson 1986, Lim et al. 1996). However, studies with red sea bream (Sakamoto and Yone 1976), yellow tail (Ikeda et al. 1973) and common carp (Sakamoto and Yone 1978b) showed that the growth of these fish was not affected by iron-deficient diets. In contrast, Suzuki et al. (1982) reported a significant improvement in growth of eel fed a standard eel feed supplemented with 250 mg iron/kg from iron amino acid chelate. The present results indicate that supplementation of iron to the basal diet is necessary to obtain normal growth of abalone, *H. discus hannai*.

Many studies examining the dietary elemental requirements of aquatic species have shown that depressed whole-body or tissue levels of essential elements could result from insufficient dietary intake (Lovell 1978, Ogino and Yang 1978, Ogino and Yang 1979, Gatlin et al. 1982, Wilson et al. 1982, Paripatananont and Lovell 1995). Baker (1986) also indicated that studies on the mineral

TABLE 4.

Carcass composition in abalone fed various levels of supplemental iron from $FeSO_4$ or FeMet for 16 weeks (means ± SD, n = 3).

Supplemental Iron* (mg/kg)	Moisture (%)		Protein (%)		Lipid (%)	
	$FeSO_4$	FeMet	$FeSO_4$	FeMet	$FeSO_4$	FeMet
0	77.06 ± .25	77.06 ± .25	52.90 ± .10[a]	52.90 ± .10[a]	7.29 ± .16	7.17 ± .13
10	77.20 ± .17	76.01 ± .20	53.60 ± .18[b]	53.37 ± .10[b]	7.42 ± .10	7.31 ± .10
20	77.10 ± .28	77.18 ± .15	53.37 ± .10[ab]	53.86 ± .14[b]	7.39 ± .10	7.34 ± .21
30	76.29 ± .18	76.93 ± .10	53.62 ± .11[b]	53.20 ± .13[ab]	7.61 ± .10	7.49 ± .23
60	77.14 ± .28	77.28 ± .25	53.56 ± .20[b]	53.78 ± .11[b]	7.49 ± .11	7.34 ± .10
120	77.33 ± .11	77.21 ± .18	53.59 ± .13[b]	53.42 ± .10[b]	7.43 ± .10	7.33 ± .23
200	77.04 ± .23	77.08 ± .17	53.33 ± .20[ab]	53.63 ± .16[b]	7.41 ± .17	7.47 ± .20
ANOVA						
F value	.4981	.8516	6.7800	17.6268	1.7016	.4072
P value	.7929	.5696	.0118	.0007	.2510	.8531

* The basal diet contained 24.9 mg of iron/kg diet, and the measured total dietary iron levels are the same as those in Tables 2 and 3, respectively.
[a–b] Means in the same column sharing a common superscript letter were not significantly different ($P > .05$) as determined by Tukey's test.

TABLE 5.

Ash and iron, manganese content in the shell of abalone fed graded levels of dietary iron from $FeSO_4$ or FeMet for 16 wk (means ± SD, n = 3).

Supplemental Iron* (mg/kg)	Ash (%)[a]		Iron (μg/g)[b]		Manganese (μg/g)[b]	
	$FeSO_4$	FeMet	$FeSO_4$	FeMet	$FeSO_4$	FeMet
0	74.53 ± .10	74.53 ± .10	128 ± 9.41	128 ± 9.41	9.12 ± .78	9.12 ± .78
10	74.69 ± .16	74.56 ± .10	119 ± 7.94	130 ± 4.82	8.74 ± .91	8.49 ± .73
20	74.56 ± .10	74.60 ± .10	135 ± 12.52	123 ± 10.56	9.04 ± .46	9.19 ± .95
30	74.60 ± .10	74.52 ± .16	128 ± 10.18	118 ± 12.18	8.86 ± .52	8.46 ± .78
60	74.57 ± .12	74.66 ± .10	120 ± 9.08	125 ± 7.75	9.17 ± .54	9.02 ± .56
120	74.50 ± .13	74.55 ± .10	132 ± 7.82	134 ± 6.96	8.47 ± .90	8.77 ± .48
200	74.52 ± .20	74.46 ± .12	126 ± 5.65	129 ± 11.03	8.68 ± .77	9.11 ± .79
ANOVA						
F value	.4811	.4210	.6730	.6584	.5782	.5956
P value	.8043	.8441	.6785	.6818	.7144	.7008

* The basal diet contained 24.9 mg of iron/kg diet, and the meaured total dietary iron levels are the same as those in Tables 2 and 3, respectively.
[a] Dry weight basis.
[b] Wet weight basis.

requirements of animals should include measurement of body stores of the test element. Mineral analyses at the end of the feeding trial indicated that soft-body iron concentrations of the abalone increased linearly until the dietary iron reached 66.78 mg/kg for FeMet (Fig. 4A), and 64.55 mg/kg for $FeSO_4$ (Fig. 4B). The reduced iron reserves were becoming depleted and deficiency signs would most likely become apparent if those diets were fed for an extended period of time. Therefore, the soft-body iron concentration was also the responsive criterion for estimating the dietary iron requirement of the abalone. However, similar responses were not observed in shell iron concentrations of the abalone. This suggests that shell iron deposition of the abalone was not a useful criterion for determining the iron requirement of the abalone, especially when the experimental duration is not long enough. Aquatic shellfish have special formation mechanisms for biomineralization of their hard tissue. Sakai (1980) found that the accumulation of organic acids in the rearing water could lead to severe shell erosion in the young abalone, and cause the shell to split along the respiratory apertures. Chen (1989) reported that there was a marked depression in calcium and zinc concentration in split of *H. diversicolor supertexta*. These results, along with the data obtained in the present study, implies that the rearing water quality, such as pH, perhaps play a more significant role than the dietary mineral concentration in the diets in shell mineralization and shell mineral deposition of abalone.

Interaction among minerals or nutrients can decrease intestinal absorption of inorganic nutrients. Johnson and Korynta (1992) indicated that excess iron decreased the absorption of manganese in rats. Results of mineral analyses in the present study showed significantly reduced manganese deposition in the soft-body of the abalone fed diets containing high levels (≥100 mg/kg) of iron from $FeSO_4$. However, this effect of excess iron on utilization of manganese was not found when using FeMet as the iron source. The probable explanation is that when the mineral is bound in chelated form, the interaction between the chelate and other minerals or other compounds is prevented to a certain extent.

The minimum level of dietary iron for juvenile abalone slightly varied with iron sources and criteria. Data from growth, and soft-

TABLE 6.

Ash and iron, manganese content in the soft body of abalone fed graded levels of dietary iron from $FeSO_4$ or FeMet for 16 wk (means ± SD, n = 3).

Supplemental Iron* (mg/kg)	Ash (%)[1]		Iron (μg/g)[2]		Manganese (μg/g)[2]	
	$FeSO_4$	FeMet	$FeSO_4$	FeMet	$FeSO_4$	FeMet
0	11.51 ± .15	11.51 ± .15	312 ± 12.86[a]	312 ± 12.86[a]	6.58 ± .78[b]	6.58 ± .78
10	11.54 ± .12	11.59 ± .10	376 ± 10.94[b]	359 ± 9.38[b]	6.46 ± .97[b]	6.28 ± .97
20	11.50 ± .10	11.55 ± .10	426 ± 7.97[c]	419 ± 8.58[c]	6.57 ± 1.02[b]	6.40 ± 1.15
30	11.57 ± .10	11.49 ± .18	458 ± 18.41[cd]	461 ± 18.12[cd]	5.98 ± .76[b]	6.06 ± .86
60	11.49 ± .10	11.59 ± .14	519 ± 9.74[d]	530 ± 17.74[d]	5.77 ± .94[b]	6.10 ± .81
120	11.48 ± .17	11.57 ± .10	528 ± 12.66[d]	517 ± 10.88[d]	4.89 ± .72[a]	6.29 ± .66
200	11.58 ± .11	11.56 ± .13	514 ± 18.25[d]	526 ± 13.13[d]	4.92 ± .74[a]	6.15 ± .78
ANOVA						
F value	.1428	.2089	54.2785	56.3374	37.8865	.4186
P value	.9487	.9625	.0000	0.0000	.0000	.8627

* The basal diet contained 24.9 mg of iron/kg diet, and the measured total dietary iron levels are the same as those in Tables 2 and 3, respectively.
[1] Dry weight basis.
[2] Wet weight basis.
[a–d] Means in the same column sharing a common superscript letter were not significantly different ($P > .05$) as determined by Tukey's test.

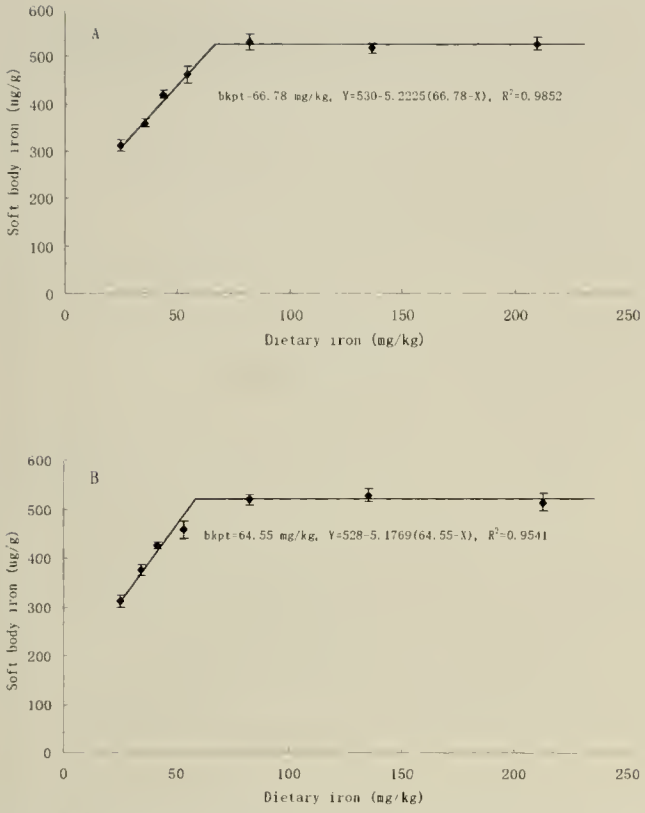

Figure 4. Regression of soft-body iron concentration (μg/g, wet weight basis) on dietary iron levels and breakpoints (bkpt) in the lines for juvenile abalone fed the diets containing graded levels of iron either from methionine (FeMet) (A) or from iron sulfate ($FeSO_4$) (B) for 16 wk.

body iron concentration showed that about 63–67 mg/kg of dietary iron from FeMet could maintain optimum growth and soft-body iron deposition. When using $FeSO_4$ as supplemental iron source, the minimum level of dietary iron was 60–65 mg/kg. We therefore recommended that dietary iron requirement of juvenile abalone is 65–70 mg/kg. This estimated requirement is in agreement with Lall and Hines (1987), who determined that the dietary iron requirement of Atlantic salmon was 60 mg/kg. However, this requirement is higher than those reported for channel catfish (30 mg iron/kg; Gatlin and Wilson 1986, Lim et al. 1996), and lower than those reported for eel (170 mg iron/kg; Nose and Arai 1987), and sea bream (150 mg iron/kg; Sakamoto and Yone 1978a).

Studies with mammals have shown that chelation of minerals to amino acids may increase their absorption rate in the intestine (Ashmead 1992). He indicated that the higher bioavailability of amino-acid-bound trace elements to animals is because chelation protects the element from forming insoluble complexes in the digestive tract and facilitates mineral transport across the intestinal mucosa. He also suggested that the chelate could remain intact until it reaches the site in the body where the element is needed. Paripatananont and Lovell (1995) indicated that the relative bioavailabilities of ZnMet, with $ZnSO_4$ as the standard, were 352% for weight gain and 305% for bone zinc deposition in egg-white diet in channel catfish. Our previous study also showed that the bioavailability of ZnMet is approximately three times as high as that of $ZnSO_4$ to juvenile abalone, *H. discus hannai* (Tan and Mai 2000). In the present study, however, we found that FeMet were equally effective as $FeSO_4$ for improving growth and decreasing mortality in abalone. This is in agreement with Lim et al. (1996), who reported that iron methionine and iron sulfate were equally effective in preventing anemia in channel catfish. The reason for the difference between the efficacy of chelated zinc and chelated iron as mineral sources for animals is that calcium and/or phosphorus bind dietary zinc and decrease its absorption through the intestinal mucosa (Lewis et al. 1994), but iron is not similarly inhibited; therefore, chelation may be not as effective for iron as for zinc (Lim et al. 1996).

Results of the present study indicated that a level of 65–70 mg of iron/kg either from FeMet or from $FeSO_4$ was sufficient for growth and tissue iron deposition in the abalone. Both iron methionine complex and iron sulfate heptahydrate were equally utilized by abalone, *H. discus hannai*. Further studies should focus on the responses of the abalone to dietary iron from the two sources of iron, using other criteria, such as immune parameters and hematological values.

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POTENTIAL DISPERSION OF REPRODUCTIVE PRODUCTS AND LARVAL STAGES OF ABALONE (*HALIOTIS SPP.*) AS A FUNCTION OF THE HYDRODYNAMICS OF BAHIA TORTUGAS, MEXICO

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ABSTRACT Field observations of currents and water mixing were made in autumn 1996, at four coastal sites close to Bahia Tortugas, on the central part of the Baja California Pacific coast, to evaluate the influence of the hydrodynamics on the transport of abalone larvae (*Haliotis spp.*). Current measurements and full-scale Lagrangean experiments on surface-water transport were carried out during the main spawning season of this genus in the area. Tidal currents seem not to be the dominant factor, but instead both wind- and wave-driven currents appear to be the most important factors for larval transport in this coastal area. Additional echo sound and aerial surveys confirmed that the reef topography and kelp beds attenuate current velocity. The hypothesis of larval dispersion is that during a typical 3- to 5-day pelagic period, larval and postlarval stages might be retained primarily in areas close to parental reefs. Flushing time in Bahia Tortugas was evaluated as five semidiurnal tidal periods. Sufficiently intensive currents at the mouth of the bay (up to 25 cm/sec) may complicate larval interchange between northern and southern vicinities of the bay.

KEY WORDS: abalone, *Haliotis*, dispersal, larvae, hydrodynamics, transport

INTRODUCTION

Currently, abalone (*Haliotis spp.)* catch off the Baja California Penninsula coastal zone is so limited that the stability of the fisheries is threatened. Catch has dropped from 6,000 tons annually in 1950 to around 600 tons (meat weight) in 1997 (Semarnap 1997). Some authors attribute this scarcity to overfishing and consequent low levels of recruitment (Guzmán del Próo 1992, Prince and Guzmán del Próo 1993), which in turn has led to strict management measures based on a quota system for each fishing zone (Ramade-Villanueva et al. 1998).

A crucial consideration in the management and administration of these fisheries is a clear understanding of the concept of a unit stock (Shepherd and Brown 1993), and this requires knowledge of larval dispersal. Two different hypotheses on larval transport have been proposed for abalone. The first postulates that the larvae can be transported for long distances from their parent reef and supply other distant reefs (Forster et al. 1982, Tegner and Butler 1985). The second establishes that larval interchange between reefs is limited and that each reef is a small independent stock that replenishes itself, with very limited larval mixing between neighboring reefs (Prince et al. 1988, McShane and Smith 1991). The first hypothesis implies a management policy that would cover long stretches of coastline, whereas the second would require individual management of each reef.

Information that could help to solve this question is a knowledge of the type of circulation and hydrodynamic patterns in the coastal areas, where abalone are found on locally isolated rocky reefs parallel to the coast from the intertidal zone down to 20-m depths (average). Large abalone concentrations are found among giant kelp beds (*Macrocystis pyrifera*) and abundant algal vegetation that serves as their habitat and food.

The reproductive period of *Haliotis fulgens* and *H. corrugata* is from late summer, during fall and the onset of winter (Sevilla 1972, Belmar-Pérez and Guzmán del Próo 1992, García and Ortíz 1992). Spawning for boths species in Tortugas begin in summer (August) and last trough the autumn, ending in December or January (Andrade 1971, Guzmán del Próo unpublished data); however, the peak spawning ocurs over a more restricted period (October through November). Larval metamorphosis, trocophore to veliger stage, takes place in the water column, where larvae float for some 3 to 5 days and for a maximum of 15 days before the settling larvae attach to the bottom and begin their benthic life (Leigthon 1974). Recruitment into the fishery depends to a large degree on high settling success on a suitable rocky substrate (McShane 1996).

Larval dispersal depends on the coastal topography and coastal hydrodynamics adjacent to abalone habitat (Shepherd et al. 1992). Some authors propose larval dispersion is limited to tens or hundreds of meters, with almost immediate settlement taking place in the vicinity of the parent stock (Prince et al. 1988), but others suggest that larvae are transported for kilometers, depending on the current regime, wave action, and storms (Sasaki and Shepherd 1995). Since juveniles and adults are practically sedentary organisms whose displacements do not exceed tens of meters (Shepherd 1973, 1986), larval supply and postlarval settlement turns out to be a critical phase for recruitment and subsequent abundance of abalone adults. Although there are uncertainty of the relationship between larval supply and recruitment in abalone (McShane 1995), it is only in this manner that adult abalone populations can replace themselves and exchange genetic material. A larval exchange on large distances or confined areas, has important implications for the management of this fishery.

In this article, we analyze the results of hydrodynamic studies made in autumn 1996 at selected abalone reefs close to Bahia

Tortugas, where current velocities and wave action were measured by Eulerian and Lagrangean methods. The objective was to evaluate, during the peak spawning season, the influence of the hydrodynamics on the transport of abalone larvae, and the potential larval conectedness between the abalone reefs located at north and south of Bahia Tortugas. This allowed us to formulate preliminary hypotheses regarding the potential larval dispersion of *Haliotis fulgens* and *H. corrugata*, which inhabit this zone.

AREA OF STUDY

The zone under study covered the area where abalone reefs are located, to the north and south of Bahia Tortugas, including the bay proper. This coastal zone forms part of the area which exploits the Bahia Tortugas Fishing Cooperative (Fig. 1).

Four sites were selected for study: La Colifloruda (Station I, 12 m depth) to the north of the bay; the mouth of the bay (Station II, 16 m depth); La Pinta (Station III, 10 m depth), to the south of the bay; and La Bajada (Station IV, 6 m depth), inside the bay (Fig. 1). La Colifloruda and La Pinta are highly productive abalone reefs, with irregular bottom topography and are normally covered by extensive giant kelp (*Macrocystis pyrifera*) beds (Fig. 4, above, and Fig. 9). La Bajada (Fig. 1) is a shallow and protected bed of boulders with no kelp and where juvenile abalone are abundant. The mouth of the bay is a strait, where most of the water is exchanged between the bay and the ocean.

Given that the main channel of the mouth of Bahia Tortugas is normal to the NW–SE line, waves that manage to get into the bay come from the SW. Main wave energy entering the bay comes from this direction, and significant waves heights coming from the SW and W are in the range from 1.40 to 2.75 m (Secretaría de Pesca 1981). Between August to December, where is the entire spawnig season for *H. fulgens* and *H. corrugata*, winds are from the W. In November, winds blow from both the north and the west. In Bahia Tortugas the average annual winds are from the NW, and these are the dominant winds (Secretaría de Pesca 1981).

Figure 1. Study area. Sample site locations and main kelp beds (dashed) in the area of abalone reefs close to Bahia Tortugas, November 1996.

Tides in Bahia Tortugas are mixed, semidiurnal, ranging from 1.40 m to 2.00 m with a time lag to tidal measurements made with the mareograph at Guerrero Negro (30 km to the north) but is an almost perfect match with the wave-tide gauge at Isla de Cedros, located 120 km to the northwest (Secretaría de Pesca 1981).

MATERIALS AND METHODS

The field observations were made during the fall (September through November) using three methods: (1) measurements of sea level variations and horizontal current components by "Inter-Ocean" S4, current meter of electromagnetic induction placed 40 cm from the bottom at four selected stations (Fig. 1); (2) vertical profiling of currents by acoustic Doppler profiler "Sontek" (ADCP) at the same sites and at the mouth of the bay; and (3) Lagrangean experiments using fluorescent dye (Uranine) to estimate advection and diffusion. The measurements were made at tidal ebb and flood. Depth profiles were made with "Furuno" echo-sounder.

Current measurements at the fixed stations were made between November 26–30, 1996. At station I (La Colifloruda, Fig. 1), we recorded horizontal current components and sea level variations (to define the variables of tides and waves) with a 2-Hz sampling frequency. At station III (La Pinta, Fig. 1), the equipment was programmed to measure variations in the sea level for 10 min every hour with the same frequency. Currents and tides at stations II and IV (Fig. 1) were recorded with a sampling interval of 1 min.

At stations I and III devices were installed close to giant kelp beds, but not inside them, and additional ADCP profiles were made both close to the current meters as well as inside adjoining kelp beds. Water exchange between the bay and the ocean was determined by means of a series of current profiles at the mouth of the bay.

To record surface currents, dye-tracer experiments were done on September 13 and 14, 1996 and repeated on November 27, 1996 at the same four sites. The displacement and position of each one of the dye patches were determined by a boat with a "Magellan 5000" GPS. These position records were combined with aerial photos, which allowed us to determine the size of the dye patches by using the boat size as a scale reference. The boat was always located at the same point of the spot.

RESULTS

During field observations, typical wind patterns were observed in the area. Normally, the breeze pattern was recorded at the wind intensity up to 4–5 m/s, but on November 27, the wind increased over a 6-h period up to 8–10 m/s, and on the 29th, there were winds up to 12 m/s from the SE coupled with rain. This was possibly caused by the influence of hurricane Fausto, which passed at the extreme south of the Baja California peninsula

Bottom Currents and Tides at Fixed Stations

During the measurements the tide was mixed with a range of 1.9 m (Fig. 2a). Water movement at each of the stations is described below.

Station I, La Colifloruda

During all measurements, the average near-bed current was about 13 cm/sec and the direction of the current was consistently around 350° (NNW), which indicates a net transport in that direction (Fig. 2b). At this station there is no notable influence of tidal currents. Possibly, this effect can be explained by both local topography and the fact that we placed the equipment close to kelp beds where tidal currents are attenuated by the presence of the kelp. Short-period local waves and swells, in comparison to tides, cross the kelp losing little energy and develop a type of mass transport known as Stokes transport (Phillips 1980), which masks the tidal current. The direction of the transport, or at least its average value, is the same as the direction of incidental waves. The spectral density and direction diagrams of sea-level variations at the site during the experiment showed a swell of up to 2 meters coming from the NWW (Fig. 3).

Station II (mouth of the bay)

This is the only station where data obtained showed the presence of significant tidal currents. The near-bed currents at the mouth are reversible and changed direction from 130°–150° (the ebb) to 350°–360° (the flood) with a current speed of 1–2 cm/s during the high and low water and about 10 cm/s during the flood and ebb (Fig. 2c). Disturbance caused by strong west winds on November 28 led to intense wind- and wave-driven along-shore currents, masking tidal currents until November 30, when tidal influence became evident once again (Fig. 2c). Intensive winds can interrupt the "pump-style" tidal currents at the mouth of the bay and cause a current along the coast.

Station III (La Pinta)

Current velocities vary from 12 to 24 cm/sec in a constant direction of 180° (toward the south). They were not generated by tides, but were caused by another dynamic process; wind-driven flow and a current that compensates for the permanent wave transport (Fig. 2d). Aerial photography showed the existence of small-scale topographic eddies at the site (Fig. 4, bottom panel).

Station IV (La Bajada)

Here, the current meter was in operation for 43 h. We observed that the near-bottom current was not influenced by the tide. Shown in Figure 5 are four different hydrodynamic situations: (1) from 12:30 p.m. on November 26, the current had an average speed of 4 cm/s, wind was 3–4 m/s; (2) with no wind, we observed that the average currents were of low intensity and flow in all directions; (3) here, the drift currents were between 5–6 cm/s, but local winds increased their intensity up to 6–7 m/s, 200°–230° NE; and (4) here, the currents were very similar to (2). This pattern was caused to the combined effect of wind-driven currents and bottom friction, because the average depth was about 5 m.

ADCP Profiling at Fixed Stations

Figure 6 shows typical vertical profiles of currents measured on November 30 close to autonomous devices at four fixed stations in the abalone reefs. Wave action was filtered from current profiles for periods of up to 20 s.

The La Colifloruda profile (Fig. 6a) was a good match with data recorded by the current meter. Current intensification up to 30 cm/s was registered in sub-surface layer. At depth, the current speed varied from 10 to 20 cm/s. Although the current speed in the water body around the kelp beds was significant, inside them it dropped considerably.

Current profiles measured close to Station II at the mouth of the bay (Fig. 6b) show the tidal current, measured during the flood, had two-layer structure. Current speed was attenuated deeper than 8–10 m from 30–33 cm/s to 20 cm/s. Similar profiles in the opposite direction were recorded during the ebb. The profile in

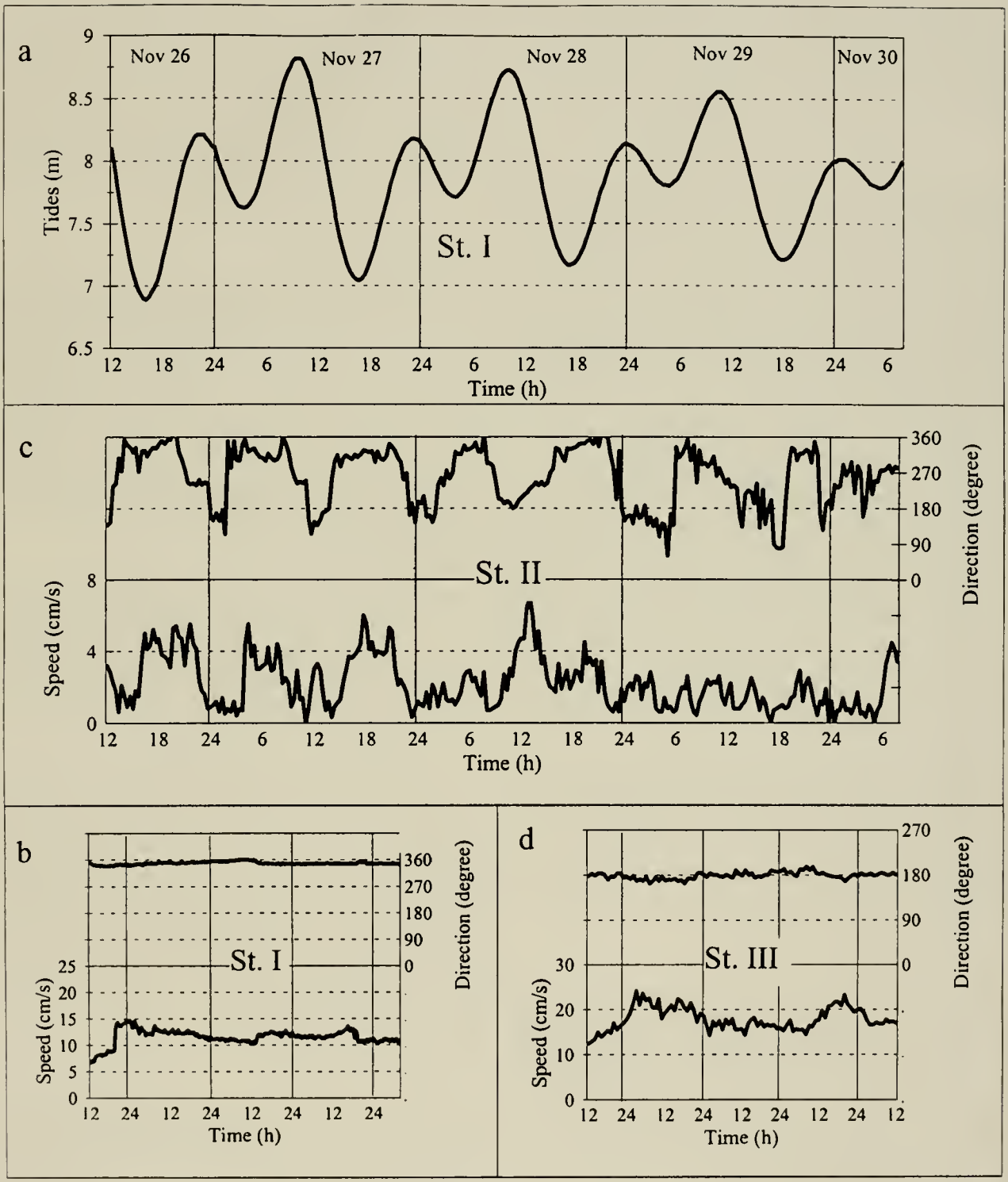

Figure 2. Tidal level variations and velocity of currents at the fixed stations: I (La Colifloruda), II (La Boca) and III (La Pinta) on November 26, 1996.

Fig. 6c, taken inside the bay close to La Bajada (Station IV), represents the vertical distribution of the current with a wind that blew at 10–12 m/s (November 30), with a gradient typical for currents induced by wind. The current profile at La Pinta (Station III) presents a more complicated structure. At depths greater than three meters speed and direction (to the S) were a good match with current recording provided by the autonomous instrument anchored at this station. However, at the surface, we note a countercurrent caused by a SW wind (Fig. 6d).

Water Exchange Between the Bay and Pacific Ocean

The series of current profiles were made during the ebb to evaluate the water exchange between the bay and ocean. Data

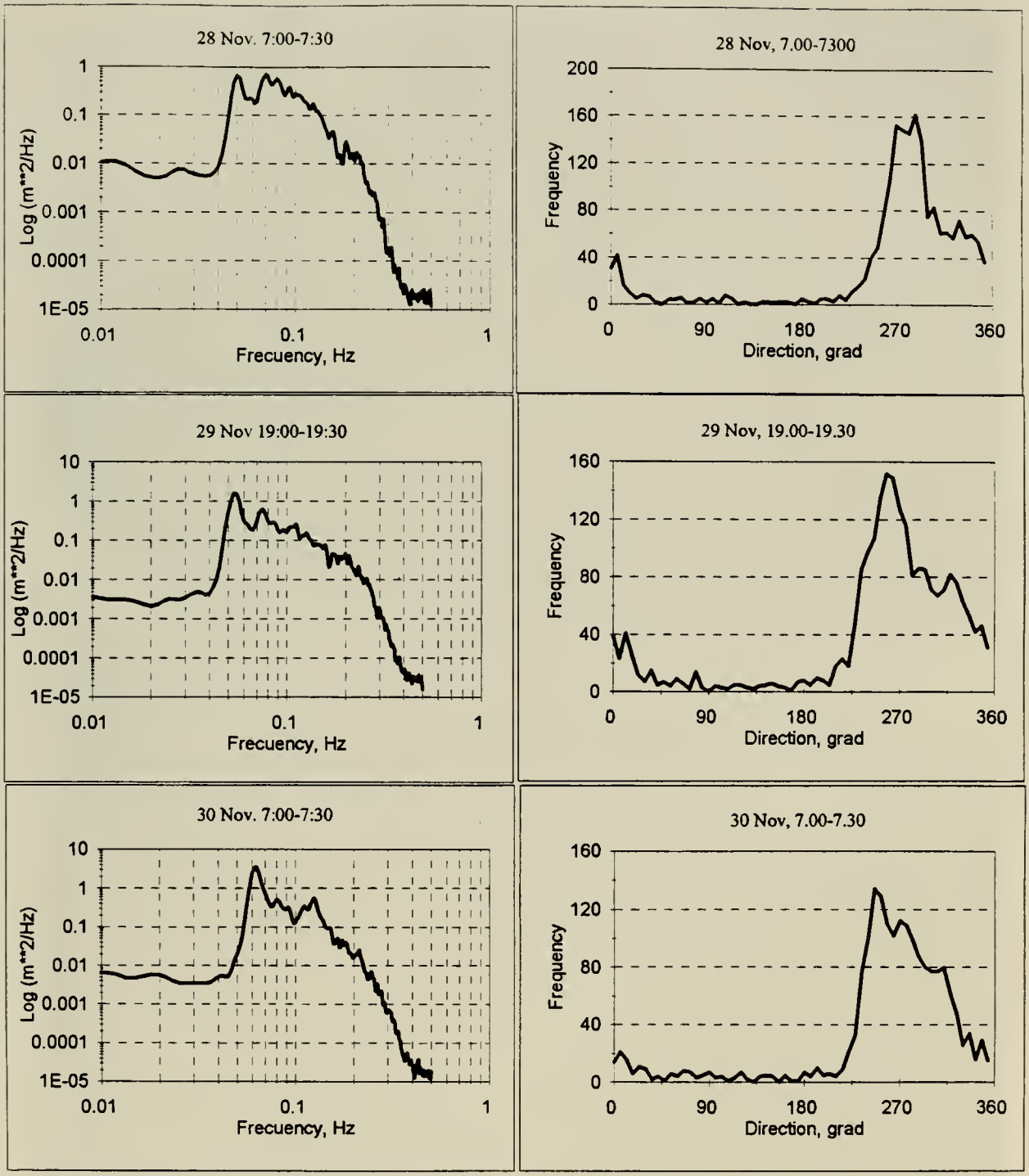

Figure 3. Typical spectral densities and directional distribution of the wave action at La Colifloruda at three different wind speed: November 28, 6–8 m/s (significant wave heights were from 0.9 to 1.0 m) and November 29 and 30, up to 15 m/sec (significant wave heights were up to 2.0 m). Main wave direction was from the West in the course of experiment time.

obtained showed most of the profiles have the shape portrayed in Figure 6b, which is typical for a channel. In addition to this, they are vertically uniform because of the tidal pressure. Near-bottom speed was slower because of bottom friction. The width of the main mouth of the bay is 3,000 m, with a depth ranging from 10–19 m. The total area of the cross-section of the mouth is 41,400 m^2 at an average tidal level (Fig. 7b). By using the vertical current profiles at the mouth of the bay (Fig. 7a), we calculated the water exchanged during a tidal cycle (November 26, 1996) was about 29 $\times 10^6$ m^3.

The bay tidal prism for the same tidal cycle was 37 $\times$ $10^6 m^3$ (the surface area of the bay is 24 $\times 10^6 m^2$, and tidal variation during this cycle was 1.58 m). The approximate volume of the bay at an average sea level is 145 $\times$ $10^6 m^3$, and the approximate time the

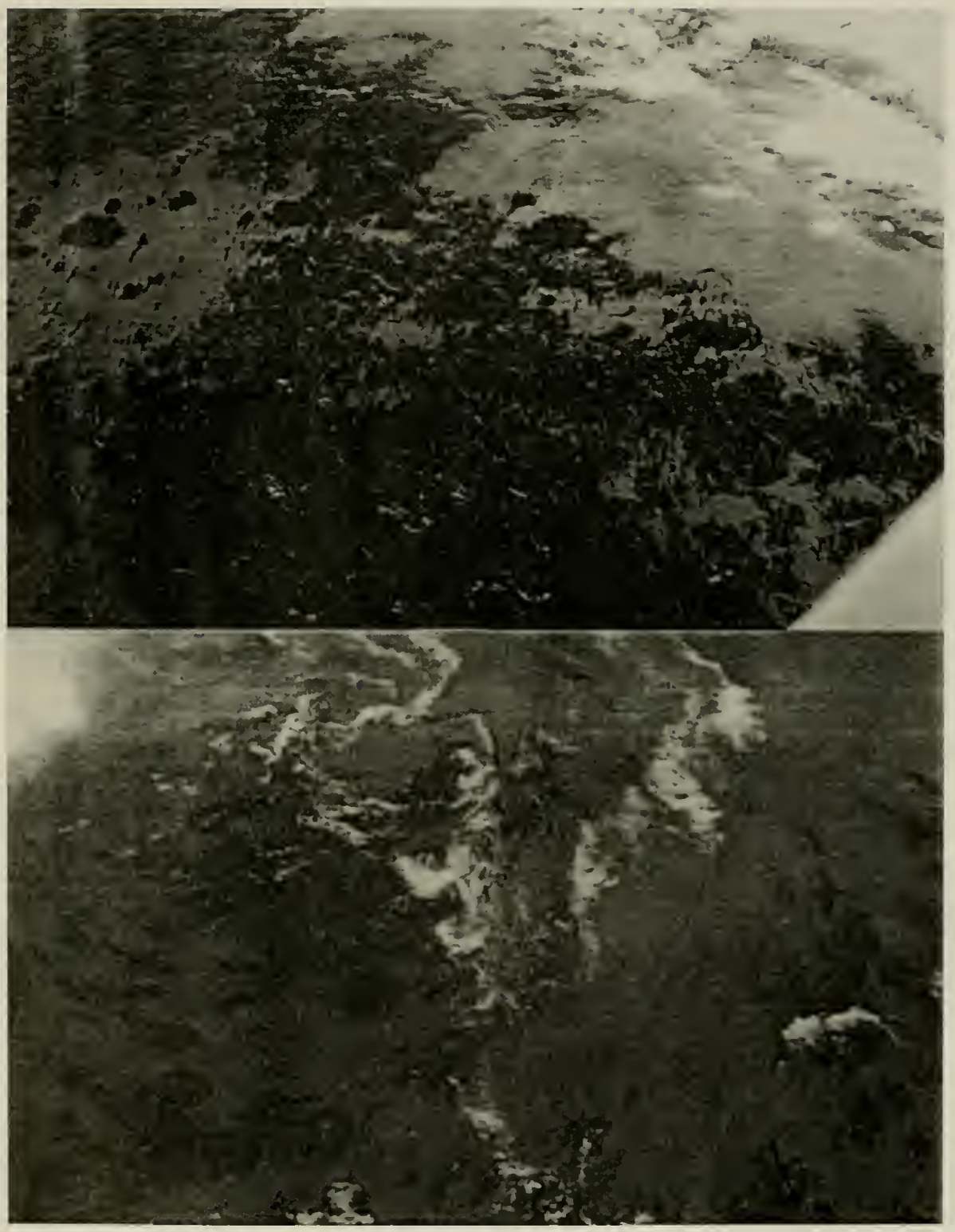

Figure 4. Kelp bed (above) and small-scale eddy (below) in the vicinity of La Pinta.

water remains within Bahia Tortugas is five semidiurnal tidal cycles.

A comparison of the tidal prism with the water exchange calculated from current profiles shows that 77% of the water was exchanged through the main channel and only 23% through the straits between the islands at the southern part of the bay, where the depth was 2 to 5 m.

Lagrangean Experiments With Fluorescent Dye

The aqueous solution of Uranina was released instantly on the surface and, because of vertical mixing, the coloring agent was dispersed to depths of 5 to 6 m during the experiment, which lasted between 2 and 3 h. The results recorded with aerial photography show the Lagrangean trajectories of dye spots and enable calcu-

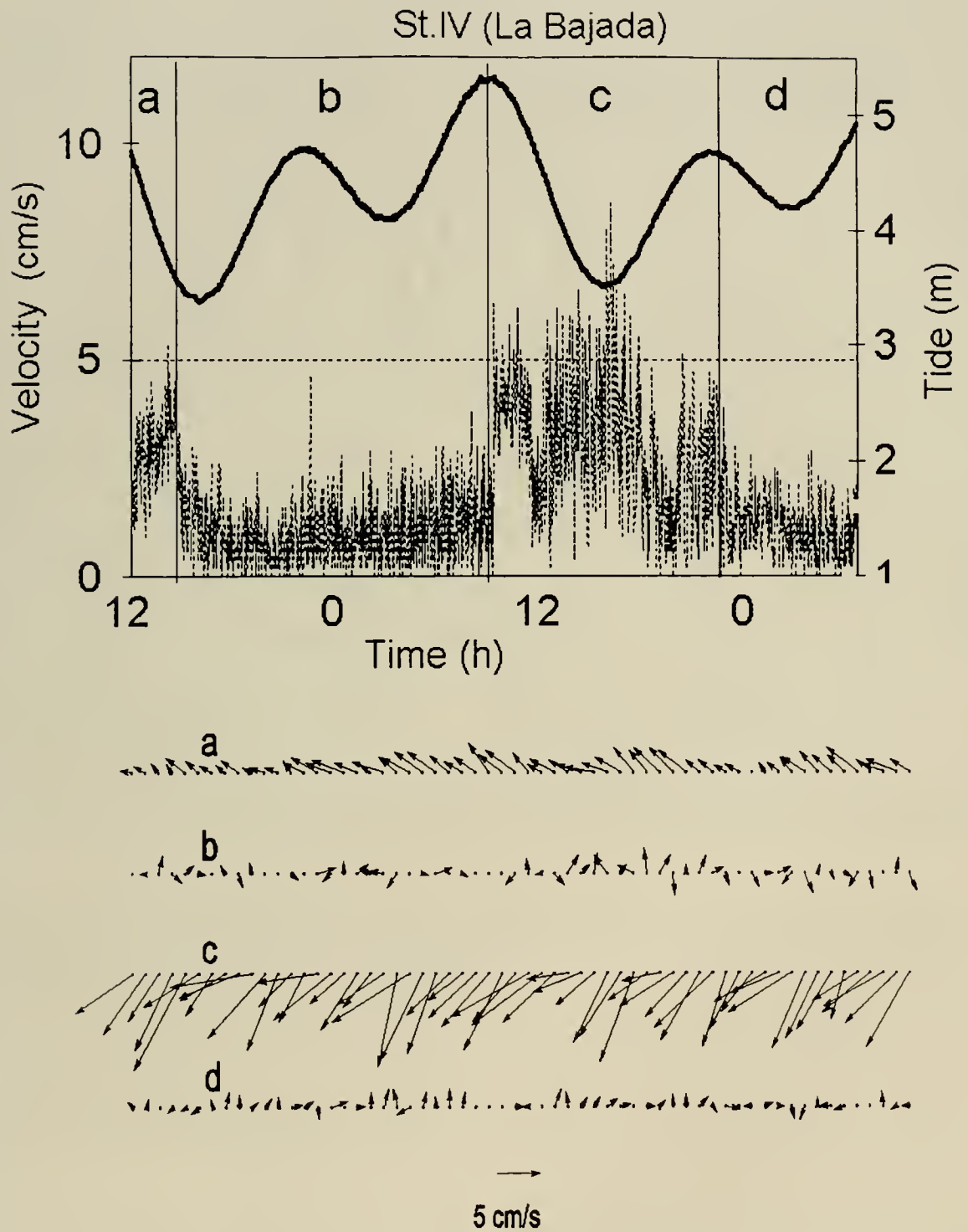

Figure 5. Currents recorded at Station IV inside Bahia Tortugas, at different times of recording (one sample per minute), November 26 through 28, 1996. Tides did not affect current pattern at this site, current velocity growth at intervals (a) and (c) was forced by wind.

lation of the average current velocity of the surface layer (Fig. 8 and Table 1). Figure 8a shows the location of the experiments. In September (Fig. 8c, ebb, and Fig. 8d, flood), inside the bay currents varied between 4 and 9 cm/sec, and trajectories of dye patches were changed according to the tidal cycle, but not significantly (patches 2, 3, and 4 in Fig. 8c; 3 and 4 in Fig. 8d). During the experiments made in November (Fig. 8b, spots 4 and 5, the ebb), the trajectories were similar to those of September. We also observed that the transport of surface water in the narrows between islands was always out of the bay, with speeds from 4 to 7 cm/s, during both the ebb tide (Fig. 8d, spots 1 and 2) and the flood (Fig. 8b, spot 3). Thus, the tides do not change circulatory patterns (in clockwise direction) in the southern part of the bay and larvae are transported into the bay through the main channel can possibly be carried out the bay through the narrows between the islands.

Experiments at the main mouth (patch 1, Fig. 8c; spots 1 and 2,

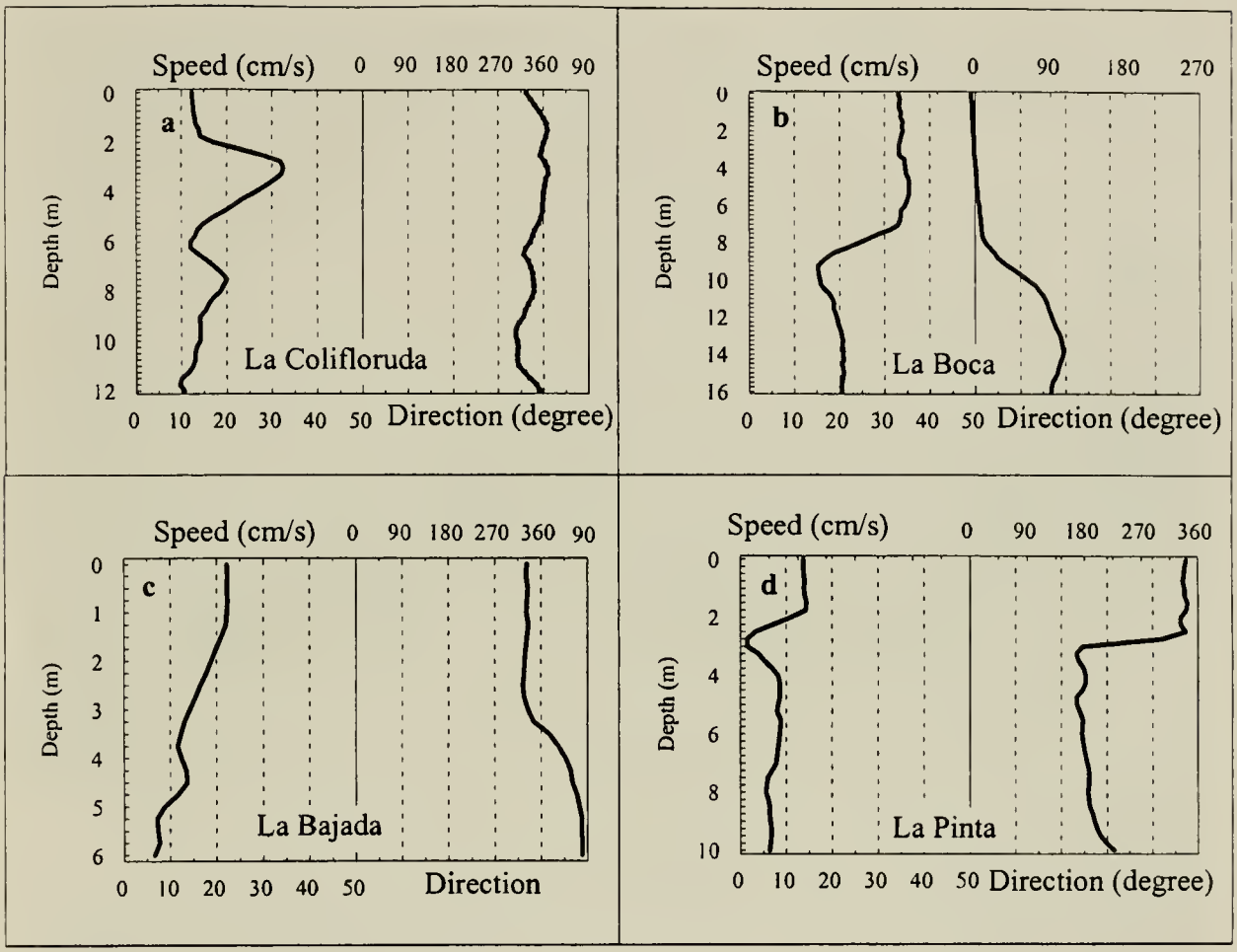

Figure 6. Typical vertical current profiles at fixed stations. Waves were filtered through these profiles with periods under 20 s (November 30, 1996).

Fig. 8b) confirm that tidal currents are between 17 and 25 cm/s and supply almost 80% of the exchange of water between the bay and the ocean.

Figure 8e shows the trajectories of the patches recorded in the northern section of the area inside the *Macrocystis* forest. In both September and November, we observed the dye transport in the kelp beds was toward the coast (to the NE), because of the wave action. This result does not match well with the data recorded by the current meter located close to the algal forest, which always detected currents toward the N (Fig. 2b). The trajectories of the patches at Playita Brava (at the extreme north of the mouth of the bay, Fig. 8e) were toward the south for both tides (flood and ebb). This effect can be explained by means of compensating currents along the coast forced by the swell. It appears the same process occurs in the southern part of the area (Fig. 8f), where the displacement of the patches is also toward the south with low speed (4–6 cm/s).

DISCUSSION

Hydrodynamic Features of the Zone

On the whole, we found that at northern and southern vicinities of the Bahia Tortugas tidal currents were not dominant, and there is a slow average water transport along the coast from the NNW to the SSE with average velocity of 4–5 cm/s. This transport was related to currents forced by wind and wave actions, but inside kelp beds along-shore water movements were attenuated and wave transport to the coast by swells prevailed, as at the sites La Colifloruda and La Pinta.

The water exchange between the bay and the Pacific Ocean, as measured by a series of vertical current profiles at the mouth (Fig. 6b), allowed us to estimate the bay flushing time as 5-semidiurnal tidal cycles, that is, 2.5 days of residence inside the bay. Therefore, the tide acts as a pump that transports water in and out of the bay at a speed up to 25 cm/s (Figs. 2c and 6b). The existence of this reversing flow is very important because its presence can interrupt the along-shore current from the north, which has a slower speed and leads to limited communication between the northern and southern groups of abalone reefs. The study shows the effects of swell (periods between 10–25 s) detected at stations I and III (La Colifloruda and La Pinta). On these external reefs, the currents were caused mainly by wind and by waves rather than by tides, especially inside kelp beds. The maximum tidal range was 1.9 m over the 4 days we examined, and the assemblage of the wave energy spectra shows that average wave heights were between 1.5 and 1.8 m, with an average period about 17 s (Fig. 3). From this, we conclude that the wave energy flux outside the bay dominated the tidal energy and that nonlinear water transport (Stokes trans-

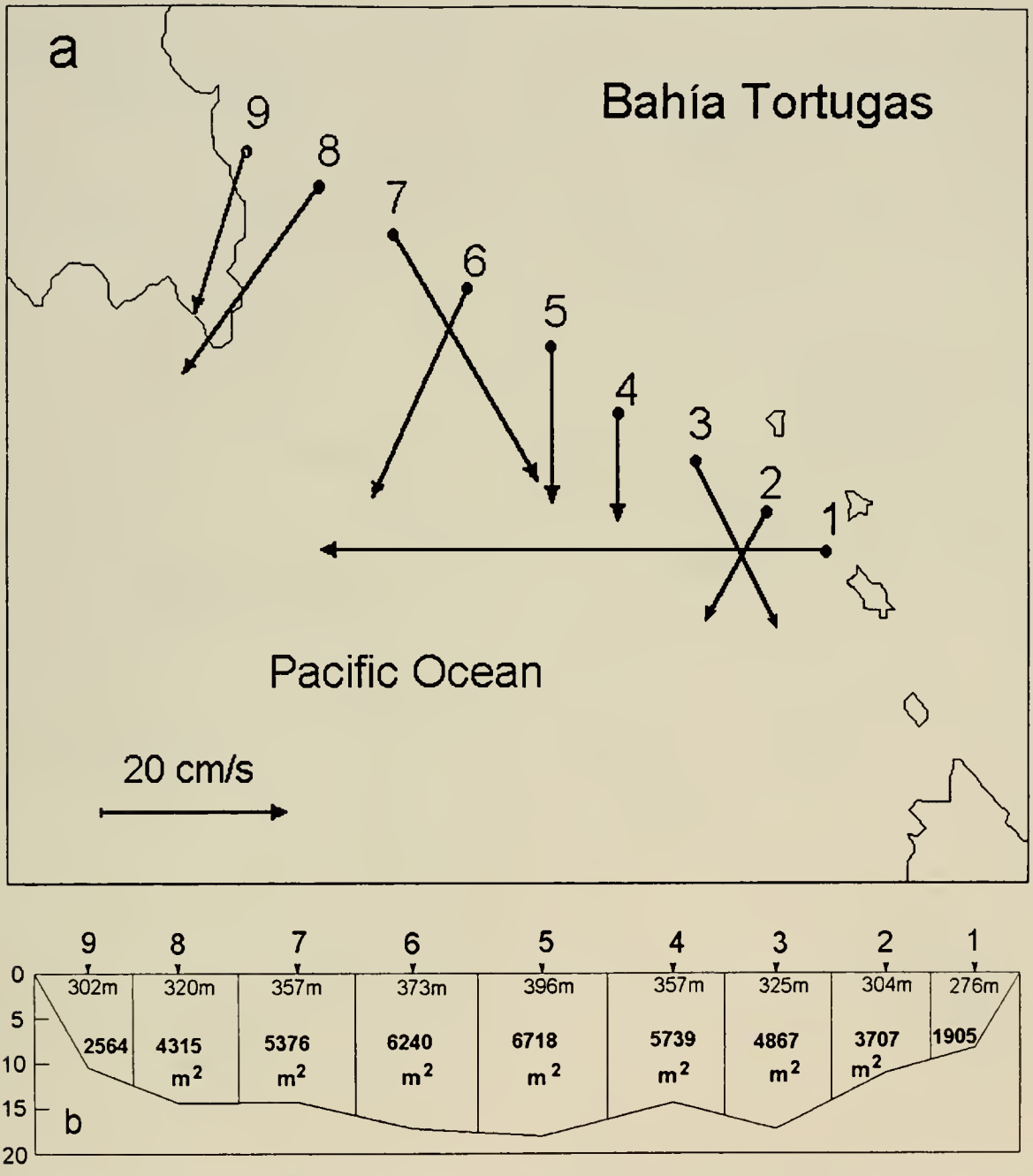

Figure 7. Scheme of water exchange calculations during the ebb: (a) Average velocity of the current at the main mouth of Bahia Tortugas on November 28, 1996; (b) vertical section of the mouth and partial area of each profile.

port) was significant because of wave action. This explains the relative constancy of direction of the current at these sites. At station IV, the origin of the currents was mostly caused by wind and bottom friction. Speed was slow, between 3 and 8 cm/s in the bottom layer, and significant only during the maximum flood and ebb. This became evident with the observed displacements of the dye patches (Figs. 8c, 8d).

At La Colifloruda, the near-bed current recorded by the autonomous current meter was persistently toward the coast, as was shown in the experiment with the dye spots (Fig. 8e), but current profiles recorded outside of the kelp forests show along-shore water movements to the SSE. Possibly this disagreement may be explained by the influence of reef topography and the presence of extensive kelp beds. It is true that inside kelp beds we found a slow water movement toward the coast caused by wave action, whereas outside of them, along-shore flow to the south was recorded. It was obvious that the presence of massive kelp beds was a key environmental element in this zone. In the water column down up to a depth of 20 m, we can see the influence of algae formations, such as kelp (*Macrocystis pyrifera*) and other groups of laminarians, such as *Eisenia, Egregia* or *Cystoceira*. Together, they form a physical barrier that can attenuate the current sometimes to one-fifth of the speed (Jackson and Winant 1983, Bernstein and Jung 1979).

Seabed topography composed of different slopes and channels

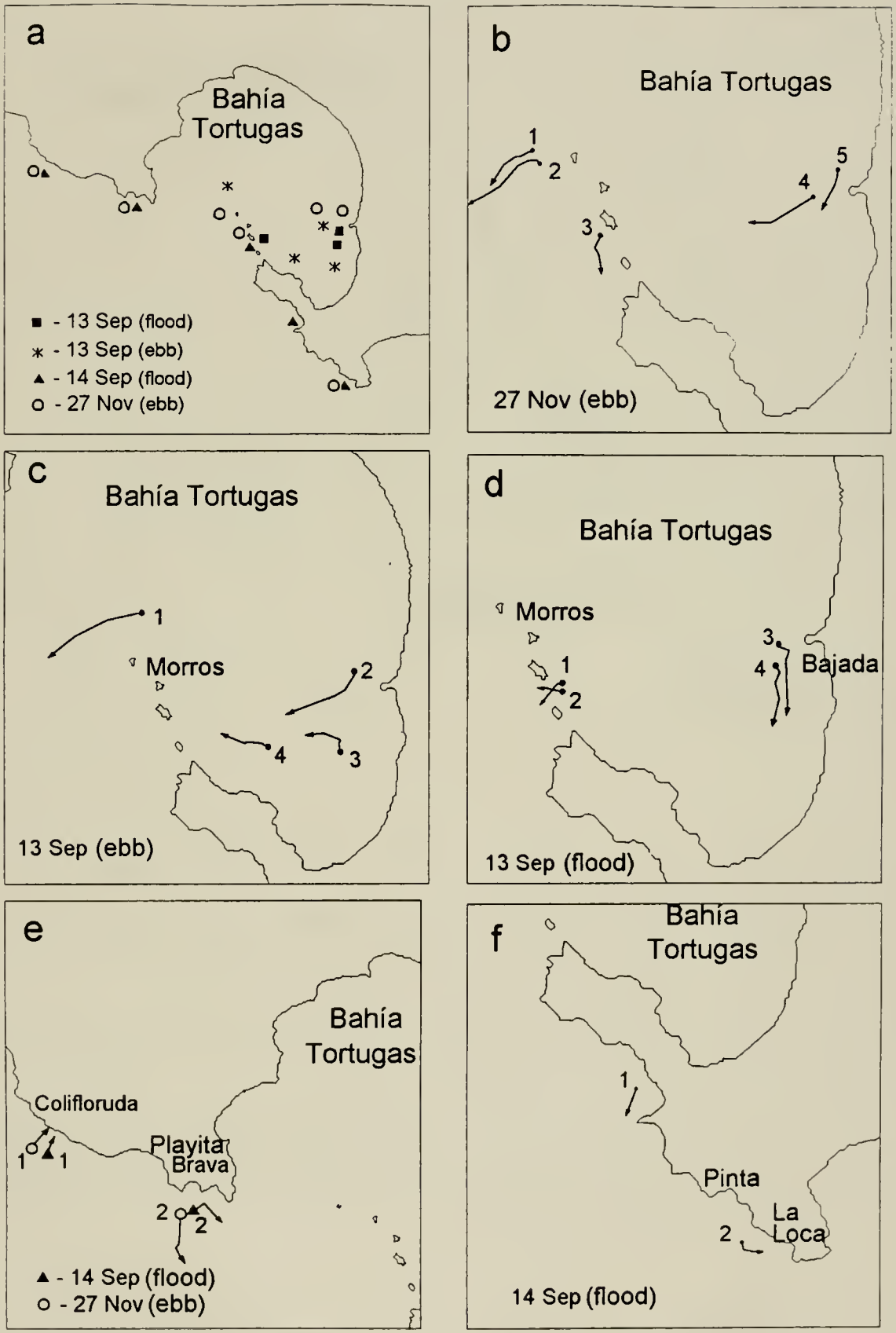

Figure 8. Lagrangean trajectories of the displacement of Uranine spots in the area of Bahia Tortugas, September 13 and 14 and November 27, 1996. Average velocities are shown in Table 1.

TABLE 1.

Average velocities of the surface transport of Uranine spots. Lagrangean experiments in Bahia Tortugas, September and November 1996.

Date (figure)	Spot number	Average speed (cm/s)	Date (figure)	Spot number	Average speed cm/s
27 Nov (8b)	1	17.2	13 Sep (8d)	1	5.0
ibid.	2	24.7	ibid.	2	4.5
ibid.	3	4.7	ibid.	3	8.3
ibid.	4	7.1	ibid.	4	5.9
ibid.	5	6.3	14 Sep (8e)	1	1.4
13 Sep (8c)	1	20.7	ibid.	2	7.6
ibid.	2	8.2	27 Nov (8e)	1	2.2
ibid.	3	4.8	ibid.	2	5.8
ibid.	4	5.7	14 Sep (8f)	1	6.2
			ibid.	2	4.6

and exposure to the coastline to swell are other influential factors (Fig. 9). Kelp beds, taken together with bottom topography and the degree of exposure, function as plankton traps (Jackson and Stratham 1981 cited in McShane et al. 1988) and explain the slow diffusion and advection of Uranine observed at reefs at La Colifloruda and La Pinta (Figs. 8e and 8f).

Within these coastal circulation processes, the on and offshore transport by internal waves or tidal bores, which is linked to the lunar cycle and the composition of diurnal and semidiurnal tidal components needs mention. Pineda (1994) has reported these waves advect water from hundreds of meters to a few kilometers from the shore, and, in spite of a limited scope of action, the importance of this transport can be critical in moving nutrients, food, and planktonic larvae in coastal waters. This type of circulation, in combination with the transport capacity of floating material and neuston through slicks and serial waves, may have a significant influence on larval recruitment in benthic systems and pelagic communities in coastal waters (Pineda 1991). We do not believe this dynamic process is continuous in time nor that it affects coastal waters shallower than 20 m, because internal waves are usually associated with the main picnocline, which was 45–50 m depth in Bahia Tortugas. So, at this site, internal waves must be reflected due to a sharp, bottom morphology. Therefore, we did not measure these types of waves, although in the coastal zone with a gentle slope internal waves must participate in the regional hydrodynamics.

Larval Dispersion Caused by Local Hydrodynamics

The main questions needed to explain the distribution of abalone banks are where and how far can larvae be transported from their point of origin? To study of the larval-transport problem, it is necessary to determine first, if some measurements of currents at certain points are sufficient to trace the trajectory of small sections of water and abalone larvae? This is far from being true. The larvae trajectories are complicated because coastal currents are not uniform and any extrapolation to assign values for distance traveled by the larvae will be only a rough estimate and will depend on the prevalence and alternation of tidal currents, wind drift, local waves, and swell at each site.

In this study, we are obviously assuming that *Haliotis* larvae or gametes are passively transported just like the inert fluorescent dye used in our experiments. Although the veliger larvae and postlarvae, before settling, have a certain ability to move in a given direction and to choose an attractive substrate for settling at the bottom (Strathmann 1974), the available evidence suggests that the veligers of abalone behave as passively transported particles (McShane 1992) and that eddies in coastal waters concentrate larvae, an observation consistent with passive transport of larvae (Tanaka et al. 1986, cited in McShane 1992).

Leighton (1974) notes that the duration of larval life is controlled primarily by temperature and varies from 4 to 15 days. He observed that larvae of *H. fulgens*, at 22–23 °C settled by the fourth day, and *H. corrugata* at 21–22 °C settling was observed as early as 3.5 days. In the reefs, the reproductive success (spawning and settling) depends on a set of hydrological conditions, since transport of larvae away from reef habitat would cause high mortality, particularly because abalone larvae have only a few days during which they are competent to settle (McShane 1992). Then, we assume the results presented here are applicable to the earliest stages of larval life of *Haliotis* before settling, i.e., the first 4–5 days when most larvae are settling. Our assumption is also supported on studies carried out at Bahia Tortugas abalone farm, where have been shown that the trocophore stage occurs after

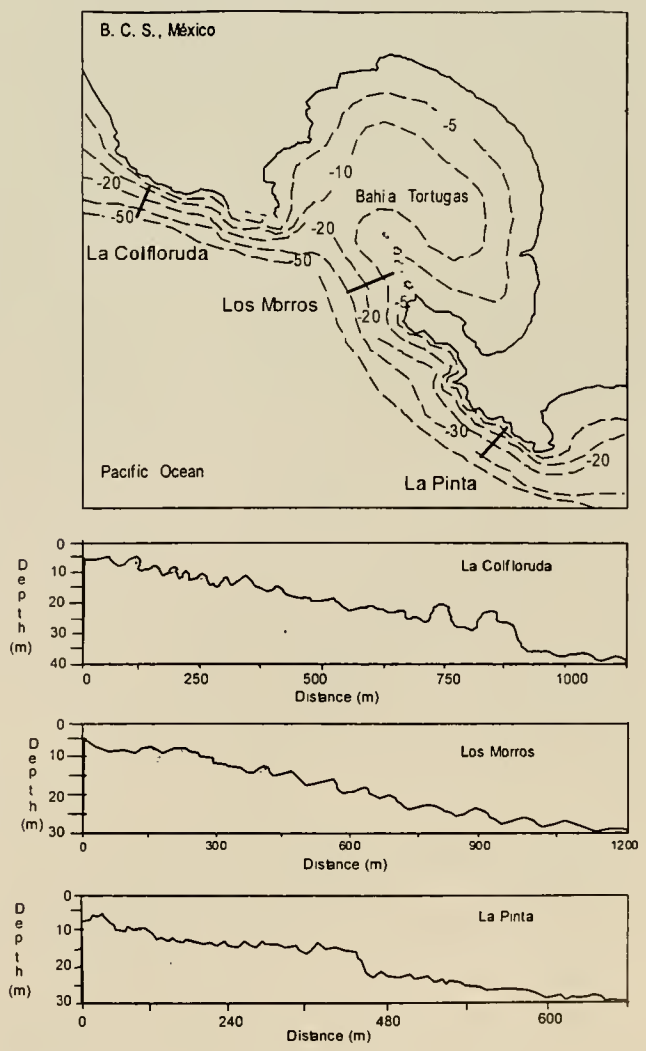

Figure 9. Bathymetric profiles of the sampling stations: (a) La Colifloruda; (b) Los Morros-La Boca; and (c) La Pinta.

12–15 h after fertilization at 16–20 °C and most settlement of veliger stages takes place 4–6 days later (Mason-Suastegui et al. 1992).

Abalone larval dispersal distances and settlement are critical components of abalone population dynamics, which continue to be a concern and for which there is not much agreement. In a broad sense, abalone are considered short-distance dispersers (Allison et al. 1998). Nevertheless, some authors state that *Haliotis* larvae can be transported several kilometers (Forster et al. 1982, Tegner and Butler 1985), in some cases up to 10 km (McShane et al. 1988). Other authors believe that the larvae settle very quickly in the neighborhood of the parent stock on a small spatial scale of tens to hundreds of meters (Prince et al. 1988). McShane et al. (1988, 1991) and McShane and Smith (1991) have supported too the hypothesis of that settlement is highly variable on small spatial and temporal scales. Some models propose that larvae may be dispersed over short or large distances depending on whether they are released near high relief reefs or in open waters respectively (McShane et al. 1988). Recently, Sasaki and Shepherd (1995) have suggested a model of larvae dispersal for *Haliotis discus* wherein the scale of dispersal is related to the intensity of the inducing storm event. Whatever the case, there is little dispute that abalone larvae are passively dispersed as considered in this study.

Our calculations of the Lagrangean transport velocities (Table 1) show that in some areas in and near Bahia Tortugas average currents can potentially transport larvae for 3 to 5 km along-shore during their pelagic cycle. The northern reefs group (La Colifloruda-Playita Brava) has 10 exploited abalone reefs, while the southern one (La Pinta-Morros) contains 12 spots (Soc. Cooperativa Bahia Tortugas 1996). The average distance between two reefs is about 1–2 km. That means that potentially larval exchange could take place between neighboring reefs. However, extensive kelp beds, small-scale topographic eddies, and onshore-wave mass transport significantly attenuate this along-shore larval flux. To be transported along the coast, larvae must leave their parent reef, but only a small number of them have a chance to do this because of small water movements inside the kelp beds. This limited number must be sufficient to maintain the larval exchange between neighboring abalone reefs of northern or southern sites near Bahia Tortugas, but it is not obvious that larvae leave their spawning area to be directly transported from the northern reefs group (La Colifloruda) to the southern one (La Pinta) or the reverse. Under normal conditions, the most probable way for larval exchange between northern and southern reefs is through Bahia Tortugas. During the flooding, larvae are transported into the bay in the northern part of the main strait by tidal flow; during the ebb, they come out through the southern straits. This is possible because the flushing time is less than the larval pelagic cycle. This scheme is valid under the hydrodynamic conditions observed, when the swells coming from the W induce long-shore average transport to the SE and tidal currents are not dominant. Thus, northern reefs donate larvae to southern ones. Under a different direction of swell, for example from the S, this scheme will be reversed. It is clear that only a small number of larvae can be transported this way. It may be that storms can only improve the larvae exchange between the northern (La Colifloruda) and southern (La Pinta) groups of abalone reefs.

CONCLUSIONS

Larval dispersion are limited and restricted to short distances in reefs whose coastal morphology and sublittoral relief are complex and covered by extensive kelp beds. This hypothesis is similar to Prince et al. (1988) and McShane and Smith (1991), who state that the larvae remain in the close vicinity of the parent stock. The results of our experiment point more to this type of dispersion than any other kind. Nevertheless, we cannot reject the possibility that more distant transport, including through Bahia Tortugas, might occur along those areas of the coastline with no massive algae formations, with gently sloping bottoms, significant tidal currents at the mouth of the bay, and with currents induced by wind and waves. Storms and strong swells that occur during certain times of the year in this area, especially winter, could lead to more distant transport such as those postulated by Sasaki and Shepherd (1995). During this time of the year, density and coverage of kelp reefs become less dense, and the current velocity should be more intense with the lesser foliage. This could also be true that for El Niño years when in Baja California all *Macrocystis* beds disappear, as occurred with the last El Niño in 1997 (Guzmán del Próo unpublished data).

We believe fisheries management should take a new approach and abalone reefs should be managed as small local and independent stock units, whose larval repopulation depends on coastal hydrodynamics, which varies from place to place. We do not believe it is advisable to continue to manage them under a policy of long stretches of coastline, as has been done to date.

ACKNOWLEDGMENTS

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THE COMBINED EFFECTS OF TEMPERATURE AND SALINITY ON GROWTH, DEVELOPMENT, AND SURVIVAL FOR TROPICAL GASTROPOD VELIGERS OF *STROMBUS GIGAS*

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ABSTRACT The precompetent period for many planktotrophic larvae of marine invertebrates is influenced by environmental factors such as food supply, temperature, and salinity. In this laboratory study veligers of the tropical gastropod *Strombus gigas* were grown in 16 temperature (20–32 °C) and salinity (30–45 ppt) combinations to examine growth, development, and survival to metamorphic competence. These environmental conditions are typical of the waters where veligers of this species naturally disperse. Temperature, and to a lesser extent salinity, can be used to estimate length of the precompetent period during the reproductive season. Veligers grown in 24 to 32 °C and 30 to 40 ppt survived well and the estimated precompetent period was 16 to 24 days long. Development was arrested and mortality was high at 20 °C regardless of salinity, and at 45 ppt regardless of temperature. To estimate dispersal potential and supply of larvae to local and distant settlement sites it is important to understand how variation in environmental conditions affects the length of larval life.

KEY WORDS: Larvae, temperature, salinity, spawning season, *Strombus gigas*, queen conch

INTRODUCTION

Larvae develop over a range of environmental conditions characterizing the locations they inhabit. In the field, the spawning season and release of larvae is usually synchronized with favorable conditions to maximize larval growth and survival (Sastry 1986). Temperature and salinity conditions affect larval growth and survival of many marine invertebrates (Kinne 1963, Kinne 1964) including crustaceans (Mene et al. 1991, Brown et al. 1992), echinoderms (Watts et al. 1982), and mollusks (Tettelbach and Rhodes 1981, Zimmerman and Pechenik 1991). Typically, temperature influences survival and growth and salinity affects survival more than growth (Tettelbach and Rhodes 1981, Nagaraj 1988, His et al. 1989, Lemos et al. 1994).

Little is known about the combined effects of temperature and salinity on growth and survival of larvae that disperse in tropical oligotrophic waters. The veliger larva of the commercially fished gastropod *Strombus gigas* (Linnaeus) disperses horizontally and vertically over a wide geographic range in the tropical coastal and open ocean oligotrophic waters of the Caribbean region (Mitton et al. 1989, Posada and Appeldoorn 1994, Stoner and Davis 1997a, Stoner and Davis 1997b, Stoner et al. 1997). The juveniles and adults of this species are found in waters that range from 17 to 32 °C, and salinities that range from 30 to 40 ppt (Davis pers. obs.). However, optimal culturing conditions for veligers of *S. gigas* are 28 to 30 °C and 35 to 37 ppt (Davis 1994a). These conditions are found in the Caribbean waters during the peak reproductive months (July to September), but each hatch of veligers may experience variations in temperature (19–31 °C) and salinity (35–50 ppt) during the 6- to 8-mo egg-laying season (Davis et al. 1984, Stoner et al. 1992, Pitts and Smith 1993, Glazer pers. comm.).

The combined effects of temperature and salinity on growth, development, and survival to metamorphic competence for veligers of *S. gigas* were tested in the laboratory. Veligers were exposed to 16 temperature (20–32 °C) and salinity (30–45 ppt) combinations found during the spawning season. These data provide insight into the upper and lower lethal limits for survival and the conditions that affect growth of *S. gigas* veligers. The results also assist in determining how temperature and salinity influence the precompetent period and supply of larvae to settlement sites throughout the spawning season.

MATERIALS AND METHODS

This laboratory experiment was designed to test the combined effects of four temperatures (20, 24, 28, and 32 °C) and four salinities (30, 35, 40, and 45 ppt) on growth rates, development, and survivorship to metamorphic competence for veligers of *S. gigas*. The experiment was a 4 × 4 factorial design, with all 16 temperature and salinity combinations tested.

The study was conducted from June to September, 1994 at the Caribbean Marine Research Center, Vero Beach Laboratory in Florida. Newly laid egg masses were collected from a spawning population near Lee Stocking Island, Bahamas (Stoner et al. 1992) and shipped to the Vero Beach Laboratory. The egg masses were incubated in a flow-through system for 4 days at ambient temperature (28 °C) and salinity (35 ppt) (Davis 1994a). On the day of hatching, several strands of the egg mass were placed in 3-L glass hatching containers.

All temperature and salinity combination treatments were not run simultaneously. Each hatch of veligers was used with one temperature treatment and all four salinity treatments (32 °C treatment was conducted in June, 28 °C in August, 24 °C the beginning of September, and 20 °C the end of September). A control treatment using optimum temperature (28 °C) and salinity (35 ppt) (Davis 1994a) was run with each hatch of veligers. The control treatment for the 20 °C-treatment was 24 °C and 35 ppt because it was difficult to maintain water at 28 °C due to heater failure. The control treatments were used to test for differences among each hatch of veligers and/or time periods. By day 20, controls from each experiment had mean shell lengths that were not statistically different ($F_{3,15} = 1.927$, $P = 0.1791$; see "Results"). This allowed for treatments to be compared statistically.

Water temperature was maintained using an incubator for the 20 and 24 °C treatments and a heated water bath for the 28 and 32 °C treatments. Seawater used in all treatments was filtered (10 μm) and sterilized with ultraviolet light. Depending on salinity of ambient seawater, salinity was lowered to 30 ppt by mixing 112 to 162 mL distilled water L^{-1} ambient seawater. To increase salin-

ity to 40 and 45 ppt, 3.8 and 7.5 g Instant Ocean L^{-1} ambient seawater was mixed together, respectively. A refractometer was used to measure salinity. Treatment water was made in the containers 24 hr in advance to allow the Instant Ocean to dissolve completely and the temperatures to adjust to treatment conditions. To assure that the use of Instant Ocean did not have a negative effect on larval growth and survival, veligers were cultured in 100% Instant Ocean made to a concentration of 35 ppt (41 g Instant Ocean L^{-1} distilled water) at a temperature of 28 °C. There were no negative effects on growth and survival for veligers grown exclusively in Instant Ocean (see "Results"). Salinity and temperature were monitored daily. Temperatures were maintained within ±2 °C and salinities were maintained within ±1 ppt.

A sample of 30 newly hatched veligers was measured to determine initial size. A dissecting microscope equipped with an ocular micrometer was used to measure shell length from apex to siphonal canal at 20× magnification. Veligers were initially stocked at 125 L^{-1} in an 800-mL transparent, polypropelene container. This concentration is similar to that used in standard aquaculture practices for this species (Davis 1994a). There were four replicate containers for each treatment. Initially, the veligers were acclimated to the temperature and salinity treatment over a period of 2 hr by gradually lowering or increasing temperature and/or salinity. Every 48 hr the veligers were placed in new water and containers. The veligers and treatment water were removed by pouring them through a submerged sieve with the appropriate size mesh (120–300 μm). A wash bottle filled with water of the corresponding temperature and salinity treatment was used to move the veligers from the sieve into the new container.

Daily veligers were fed cultured phytoplankton to satiation. They were fed exclusively Isochrysis galbana from day 0 to 10 and a mixture of *I. galbana* and *Chaetoceros gracilis* at a 3:1 ratio from day 10 to metamorphic competence (Davis 1994a). The final concentration of phytoplankton in each container was 5,000 to 10,000 cells/mL (Davis 1994a).

As the veligers grew, the number of veligers in the treatment containers was gradually reduced to 62 veligers L^{-1} on day 7, 31 veligers L^{-1} on day 13, and 12 veligers L^{-1} on day 20. This reduction in concentration was based on standard aquaculture procedures for this species (Davis 1994a). Every other day 5 veligers were removed from each replicate for measurements and developmental observations. To avoid damaging veligers during observations, they were removed carefully with a pipet and placed in a seawater-filled Petri dish. Based on velar lobe development, seven developmental stages were identified and recorded: (1) hatching; (2) two lobes; (3) beginning four lobes (4a); (4) four lobes (4b); (5) beginning six lobes (6a); (6) six lobes (6b); and (7) elongated six lobes (6c). Developmental stage at a given age was based on when 50% or more of the veligers were at that stage. The veligers removed for measurements and observations were only returned when the concentration was below the designed concentration for that day. On day 7, 13, and 20 all veligers in each replicate were observed, concentration was reduced, and dead veligers were removed and recorded to determine mortality. The treatments were run until the veligers showed the documented signs of competence such as green pigmentation on the propodium, six elongated lobes, buccal mass development, and swim-crawl behavior (Brownell 1977, Davis 1994b, Noyes 1996, Davis 2000) or until all the veligers were approaching death or had died.

ANOVA following the guidelines of Day and Quinn (1989) was used to determine if shell lengths and mortality were significantly different for veligers grown in different temperature and salinity combinations. Cochran's test was used to test for homogeneity of variances. Tukey's multiple comparison test of means was used to compare shell length and mortality data. The statistical program JMP, developed by SAS Institute, Inc. for Macintosh, was used for the statistical analyses.

RESULTS

Growth

Even though the temperature and salinity treatments were not run simultaneously and were conducted with four hatches of veligers, the mean shell length of control veligers including Instant Ocean veligers were not significantly different by day 20 ($F_{3,15}$ = 1.927, P = 0.1791; Fig. 1). Only on day 12 was there a difference. The mean shell length was statistically smaller for the 20 °C control veligers ($F_{3,15}$ = 8.431, $P < 0.05$, Tukey's test, $P < 0.05$) compared to the other control veligers. Average growth rates for veligers in the control treatments ranged from 26 to 31 μm d^{-1} and the first morphological signs of competence were observed between 24 and 26 days.

Temperature had a stronger influence on the growth rates of veligers of *S. gigas* than salinity (Figs. 2 and 3). However, veligers grown in the extreme high salinity treatment (45 ppt) grew slowly (2–19 μm d^{-1}) at all temperatures and did not show morphological signs of metamorphic competence (Figs. 2 and 3). On days 16 and 20, veligers grown at 24 and 32 °C and salinity 45 ppt had shell lengths that were not significantly different (Day 16: $F_{1,5}$ = 6.7279, P = 0.050; Day 20: $F_{1,5}$ = 5.2517, P = 0.0705; Fig. 2). On day 16, shell lengths of veligers grown at 28 °C and 45 ppt were not different from those of veligers grown at 24 °C and 45 ppt, but their shells were smaller than those of veligers cultured at 32 °C and 45 ppt ($F_{2,7}$ = 7.5294, $P < 0.05$, Tukey's test, P = 0.05; Fig. 2).

Highest overall growth rates (44–52 μm d^{-1}) were achieved for veligers grown at 32 °C and salinities 30, 35, and 40 ppt (Fig. 3).

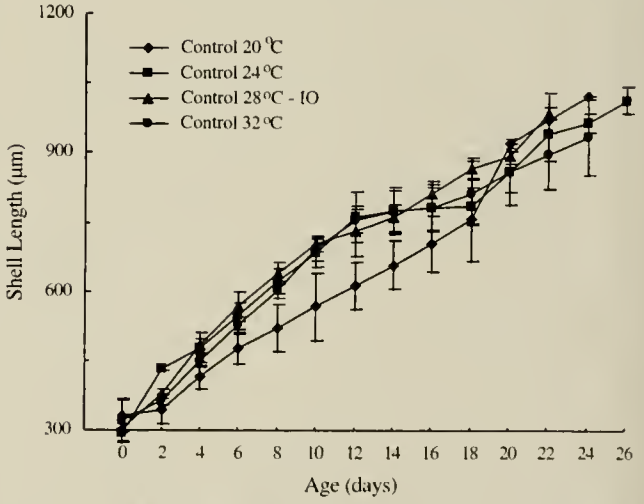

Figure 1. Growth of *S. gigas* veligers grown in control conditions. The control treatment for 24 and 32 °C treatments was 28 °C and 35 ppt. Control for 20 °C was 24 °C and 35 ppt and control for 28 °C was 28 °C and Instant Ocean (IO) mixed to 35 ppt. Data points represent means and standard deviations (n = 4 replicate containers, 5 veligers were measured from each replicate for each data point).

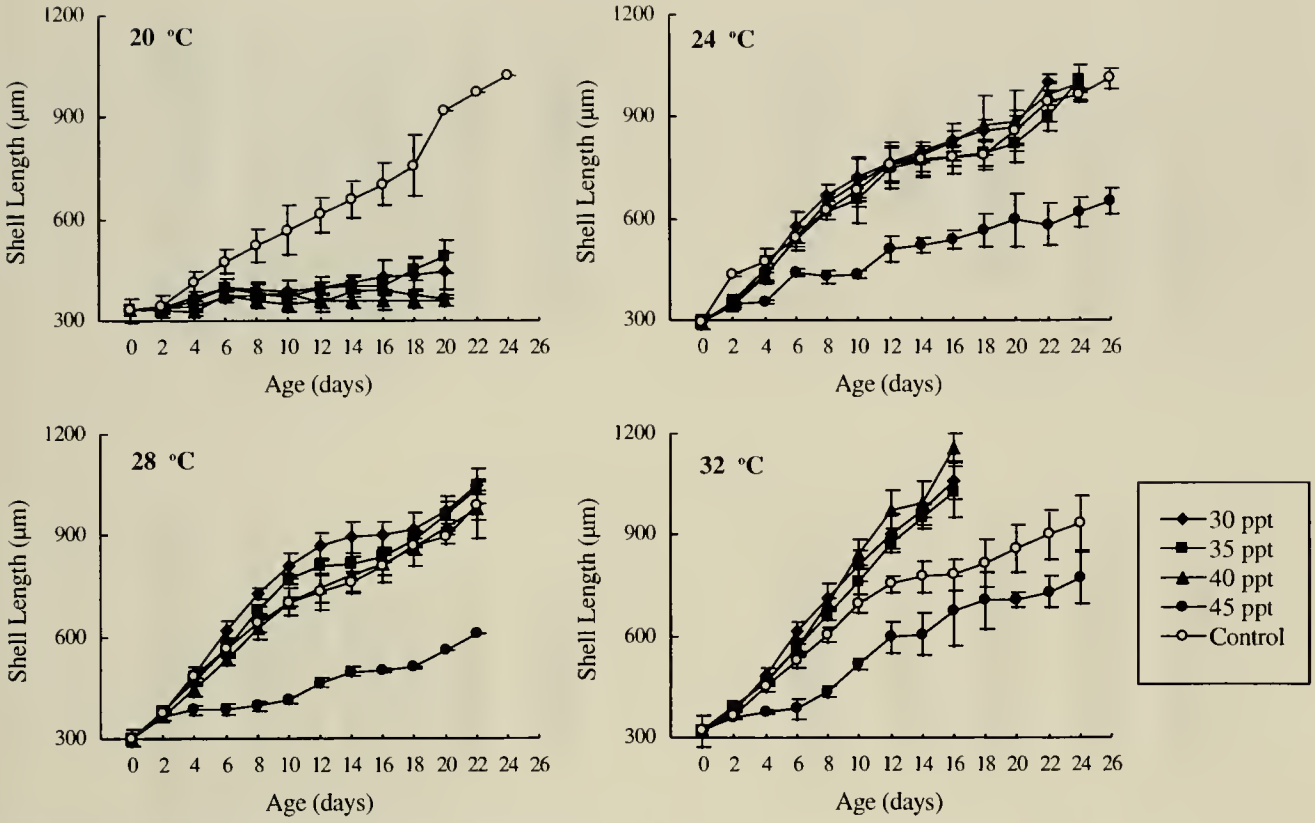

Figure 2. Growth of *S. gigas* veligers cultured in 16 temperature and salinity treatments. The control treatment for 24 and 32 °C treatments was 28 °C and 35 ppt. Control for 20 °C was 24 °C and 35 ppt and control for 28 °C was 28 °C and Instant Ocean mixed to 35 ppt. Data points represent means and standard deviations (n = 4 replicate containers, 5 veligers were measured from each replicate for each data point).

Although shell lengths on day 16 differed for veligers grown at 32 °C and 30, 35, and 40 ppt ($F_{2,9}$ = 5.0989, $P < 0.05$), veligers in the three treatments all showed morphological signs of metamorphic competence on day 16 (Fig. 2). Shell lengths were not different for veligers cultured at 32 °C and salinities 30 and 35 ppt, and 32 °C and salinities 30 and 40 ppt (Tukey's test, $P > 0.05$). Shell lengths were larger for veligers grown at 32 °C and 40 ppt than veligers grown at 32 °C and 35 ppt (Tukey's test, $P < 0.05$; Fig. 2).

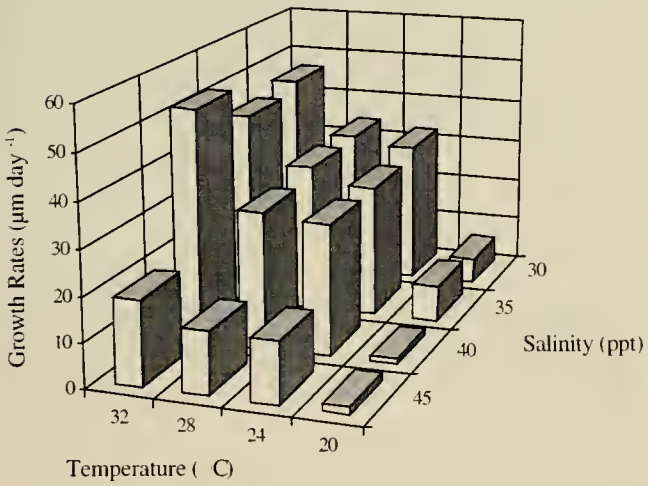

Figure 3. Overall mean growth rates of *S. gigas* veligers cultured in 16 temperature and salinity combinations (n = 4 replicate containers, 5 veligers were measured from each replicate for each data point).

Veligers grown at 24 and 28 °C and salinities 30, 35, and 40 ppt had similar growth rates (29–34 µm d^{-1}) and showed morphological signs of metamorphic competence by day 22 and 24, respectively (Figs. 2 and 3). Overall growth patterns for veligers grown in these conditions were similar; however, there were statistically significant differences in shell lengths on day 20 ($F_{5,18}$ = 4.7709, $P < 0.05$; Fig. 2). On this day, shell lengths were larger for veligers grown at 24 °C and 35 ppt and 28 °C and salinities 30 and 35 ppt (Tukey's test, $P < 0.05$), but all other shell lengths were not statistically different (Tukey's test, $P > 0.05$; Fig. 2).

The lowest growth rates (1–8 µm d^{-1}) occurred for veligers grown at 20 °C at all salinities (Fig. 3), and none of these veligers showed morphological signs of metamorphic competence. Shell lengths for veligers grown at 20 °C and all salinities were not different on day 16 ($F_{2,9}$ = 3.8884, P = 0.0607; Fig. 2). However, on day 20 shell lengths for veligers cultured at 20 °C and salinities 40 and 45 ppt were smaller than veligers grown at 20 °C and salinities 30 and 35 ppt, which had shell lengths that did not differ from each other ($F_{2,8}$ = 8.4120, $P < 0.05$, Tukey's test, P = 0.05; Fig. 2).

Developmental Stages

Veligers of *S. gigas* grown in control conditions (24 and 28 °C, 35 ppt) developed through all velar lobe stages and showed morphological signs of metamorphic competence (Fig. 4). Veligers grown at temperatures 24, 28, and 32 °C and salinities 30, 35, and

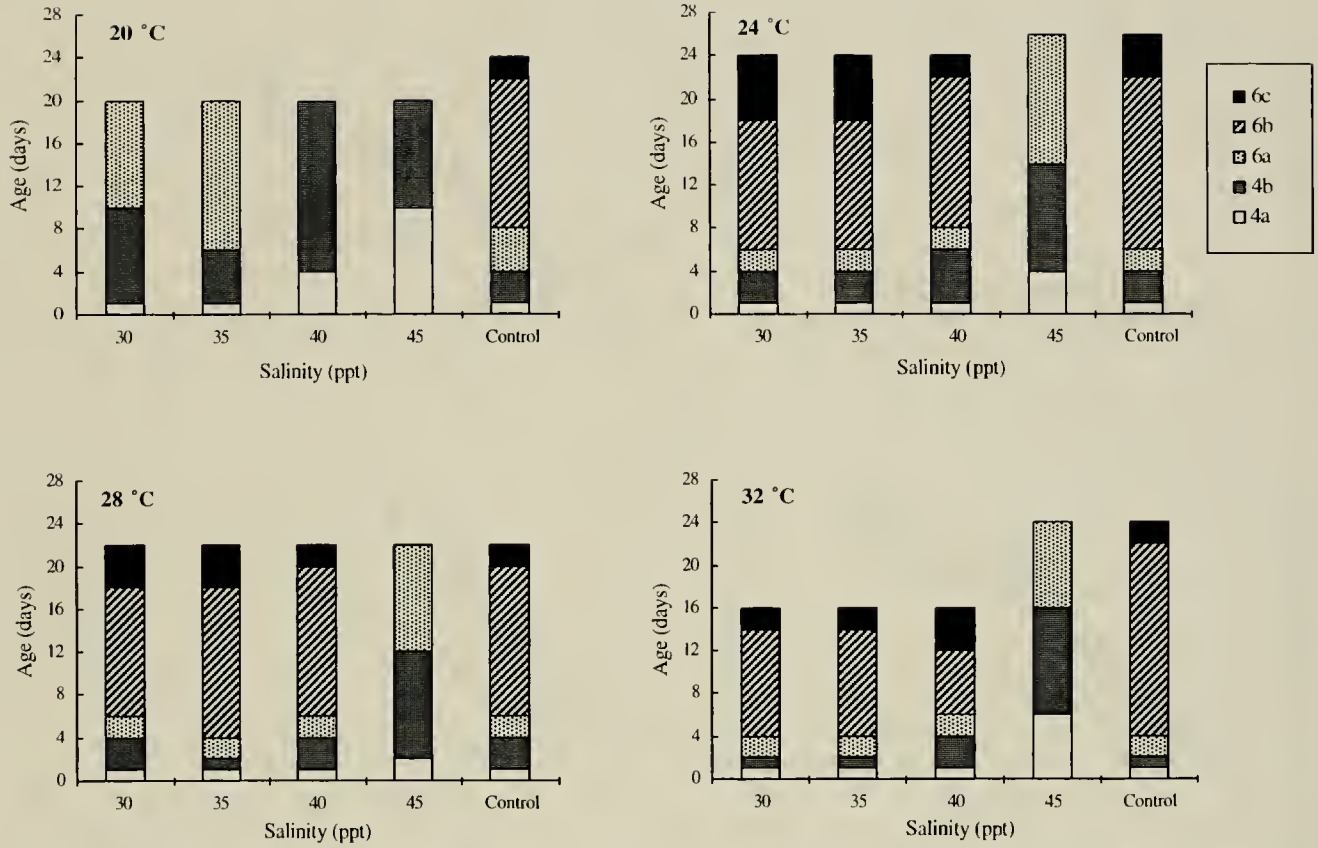

Figure 4. Developmental stages of *S. gigas* veligers cultured at 16 temperature and salinity combinations. The control treatment for 24 and 32 °C treatments was 28 °C and 35 ppt. Control for 20 °C was 24 °C and 35 ppt and control for 28 °C was 28 °C and Instant Ocean mixed to 35 ppt. Veligers hatch on day 0 with 2 lobes. The legend represents the following stages of velar lobe development: 4a, beginning four lobes; 4b, four lobes; 6a, beginning six lobes; 6b, six lobes; and 6c, elongated six lobes. The data were calculated as a percentage of veligers at each stage. The data points changed when 50% or more of the veligers developed to the next stage (n = 4 replicate containers, 5 veligers were examined from each replicate for each data point).

40 ppt developed six elongated lobes (stage 6c) and showed morphological signs of metamorphic competence (Fig. 4). Fifty percent or more of the veligers in these treatments were in the following stages: 4a, beginning four lobes for 1 to 2 days; 4b, four lobes for 2 to 3 days; 6a, beginning six lobes for 2 to 4 days; 6b, six lobes for 9 to 15 days; and 6c, elongated six lobes for 2 to 6 days prior to showing morphological signs of competence.

Development was arrested for veligers grown at all low temperature (20 °C) treatments and at all high salinity (45 ppt) treatments (Fig. 4). Veligers grown at 20 °C did not develop past 4 lobes (stage 4b) at 40 and 45 ppt and only started six lobes (stage 6a) at 30 and 35 ppt. The veligers grown at high salinity 45 ppt and temperatures 24, 28, and 32 °C did not develop beyond the beginning of six lobes (stage 6a).

Mortality

Percent mortality (mean ± SD) was low (days 0–7: 6.6% ± 2.3%, days 7–13: 3.4% ± 5.1%, and days 13–20: 1.5% ± 2.9%) for veligers grown exclusively in the Instant Ocean control treatment (28 °C and 35 ppt). Therefore, high mortality for veligers grown at 45 ppt was not attributed to the addition of Instant Ocean salts. Overall mortality was highest in all low temperature (20 °C) and all high salinity (45 ppt) treatments (Fig. 5).

At the end of the 0 to 7 day interval, percent mortality was not different and was highest for veligers grown at 20 and 24 °C and at salinity 45 ppt ($F_{1,6}$ = 0.6962, P = 0.4360; Fig. 5). Percent mortality was low and not different for veligers grown at 24 °C and salinities 30, 35, and 40 ppt ($F_{2,9}$ = 1.2521, P = 0.3313; Fig. 5). For veligers grown at 28 °C mortality was not different for veligers in 30 and 35 ppt treatments, but mortality was lower in salinity 40 ppt ($F_{2,9}$ = 6.6254, $P < 0.05$, Tukey's test P = 0.05; Fig. 5). Mortality for veligers grown in 32 °C and all salinities was high and not different among salinity treatments ($F_{3,11}$ = 1.4286, P = 0.2870; Fig. 5).

Mortality in the middle interval, 7 to 13 days, increased for veligers grown at 20 °C and all salinities and veligers grown at 45 ppt and all temperatures (Fig. 5). Percent mortality was not different for veligers grown at 20 °C and 30 and 35 ppt and temperatures 24 and 32 °C and 45 ppt ($F_{3,11}$ = 2.2068, P = 0.1447; Fig. 5). Veligers in the treatments 20 °C and salinities 40 and 45 ppt and 28 °C and 45 ppt had the highest mortality for this interval and percent mortality was marginally different ($F_{2,9}$ = 3.966, P = 0.0573; Fig. 5).

This general trend continued into the last interval, 13 to 20 days, at which time mortality was highest and not different for veligers grown at 20 °C at all salinities and 28 °C at 45 ppt ($F_{3,11}$ = 1.8573, P = 0.1953; Fig. 5). Mortality was also high and not different for veligers grown at 24 and 32 °C and 45 ppt ($F_{1,5}$ = 1.5010, P = 0.2751; Fig. 5). At the end of the 7 to 13 day and 13

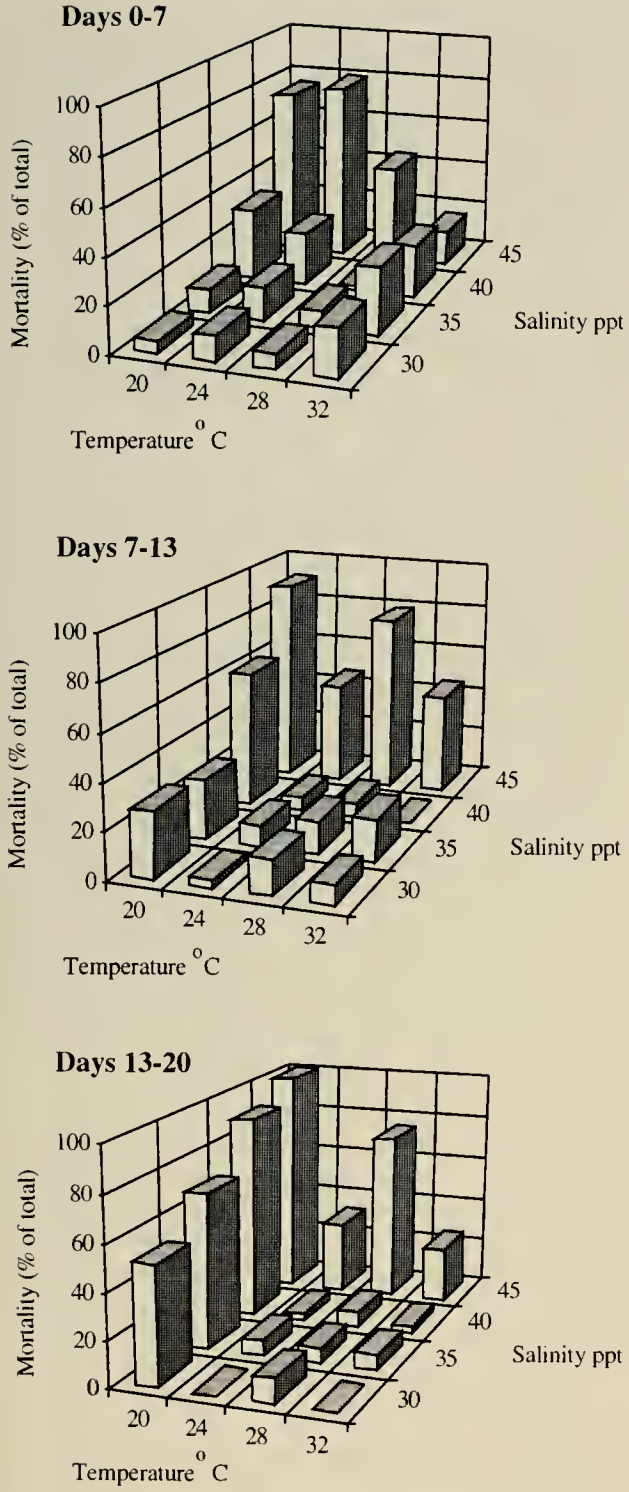

Figure 5. Percentage of mortality of *S. gigas* veligers cultured in 16 temperature and salinity combinations. Mortality was calculated as the percentage of veligers that died during each interval. Data points represent mean of 4 replicate containers and 5 veligers were measured from each replicate.

to 20 day interval, mortality was lowest and not different for veligers grown at 24, 28, and 32 °C and salinities 30, 35, and 40 ppt (days 7–13: $F_{7,24} = 1.5680$, $P = 0.1929$; days 13–20: $F_{5,16} = 0.7976$, $P = 0.5672$; Fig. 5).

DISCUSSION

In the natural environment, timing of the reproductive period and release of larvae is usually synchronized with conditions that are most favorable for maximization of larval survival and continuity of the species (Sastry 1986). Veligers of *S. gigas* showed morphological signs of metamorphic competence and maintained high survival (71%–93%) at temperatures 24 to 32 °C and salinities 30 to 40 ppt. It is not surprising that veligers grew and survived well in these conditions because these values are typically found during the reproductive season and within the geographic region where veligers naturally disperse. During the non-reproductive season, salinity stays within a narrow range, but temperature can drop as low as 17 °C in some waters where juvenile and adult *S. gigas* are found.

S. gigas veliger growth, development, and survival was influenced primarily by temperature, and salinity had a lesser effect as has been shown with other crustacean and molluscan larvae (Tettelbach and Rhodes 1982, Nagaraj 1988, His et al. 1989, Lemos et al. 1994). Therefore, temperature rather than salinity can be used to predict time to metamorphic competence during the egg-laying season. For instance, at the beginning and end of the season when temperatures are 24 to 28 °C, the precompetent period will be 22 to 24 days long. During the peak months of the season when temperatures are as high as 31 °C, the precompetent period can be just 16 days. Veliger abundance is high in the peak months not only because copulation frequency and number of egg masses laid increases 2-fold compared to the beginning and the end of the season (Davis et al. 1984, Weil and Laughlin 1984, Stoner et al. 1992, Stoner and Davis 1997a), but also because temperatures are optimal for larval growth and survival.

Other studies have indicated that veligers of *S. gigas* and *Strombus costatus* (Gmelin) develop normally at 28 °C, decrease growth at 24 °C and have 100% mortality at 32 °C (Aldana Aranda and Torrentera 1987, Aldana Aranda et al. 1989, Glazer pers. comm.). However, in this study a temperature of 32 °C provided conditions for fast growth and high survival. This is likely the highest temperature veligers encounter in most waters where they disperse. This temperature is probably near the upper physiological tolerance for these veligers, especially since most tropical marine organisms cannot survive and actively grow at temperatures higher than 35 °C (Kinne 1963). There are several examples of molluscan (Lucas and Costlow 1979, Tettelbach and Rhodes 1981, Robert et al. 1988, His et al. 1989) and crustacean (Brown et al. 1992) larvae rapidly decreasing survival and reaching a growth plateau between 30 and 35 °C. If veligers of *S. gigas* are growing at near-maximum rate at 32 °C, elevated food conditions may be the only factor capable of sustaining an increased growth rate at this high temperature (Boidron-Metairon 1995, Hoegh-Guldberg and Pearse 1995).

At a temperature of 20 °C, veliger development was arrested prior to showing any morphological signs of competence and survival rate was as low as 10% regardless of salinity. In isolated cases embryo development and larval release may occur at low temperatures (Rodriguez et al. 1991). This may be the case for embryos developing in egg masses laid in February in the Florida Keys when temperatures were as low as 19 °C (Glazer pers. comm.). However, based on the results from this study even with a successful hatching, it is unlikely that veligers would survive and develop to metamorphic competence at these low temperatures. Low larval abundance at the beginning and end of the spawning season may be due to low number of egg masses (Davis et al.

1984, Stoner et al. 1992) and decreased larval survival at low temperatures, especially in the beginning of the season.

Environmental conditions such as light are known to trigger veligers of *S. gigas* to migrate vertically (Barile et al. 1994, Stoner and Davis 1997b). Temperature may also influence migratory behavior, and the depth at which veligers migrate will depend upon their acclimation and tolerance abilities (Young and Chia 1987). The majority of veligers in the Exuma Sound, Bahamas were located above the thermocline (30 m) where temperatures of 28 to 29 °C are optimal for growth, and only on occasion veligers were found as deep as 100 m where temperature was 25 °C (Stoner and Davis 1997b). Salinity probably has little influence on vertical distribution of veligers in these same waters because salinity above the halocline (30 m) was 38 ppt and decreased only to 37 ppt at 100 m (Stoner and Davis 1997b).

In this experiment veligers of *S. gigas* showed morphological signs of metamorphic competence when salinity levels were 30 to 40 ppt and temperatures were 24 to 32 °C. However, at high salinities, such as 45 ppt, larval growth was reduced and mortality was high. It is likely that *S. gigas* veligers disperse in relatively stable salinity conditions because they are found in coastal and open ocean waters of the Florida Keys, Bahamas, and Caribbean Sea (Posada and Appeldoorn 1994, Stoner et al. 1997, Stoner and Davis 1997a, Stoner and Davis 1997b). However, on occasion veligers have been collected in shallow bank areas in the Bahamas where salinity can be as high as 43 ppt (Pitts and Smith 1993, Jones 1996) and in nearshore waters of the Florida Keys where salinity can peak at 50 ppt due to influx of hypersaline Bay water (Lapointe and Clark 1992, Fourqurean et al. 1992). It is possible that short-term exposure to high salinity in shallow waters may slow veliger growth temporarily, but long-term exposure would severely limit survival and growth to metamorphic competence. In this study the growth rate of veligers cultured at 45 ppt ranged from 2 to 19 $\mu m\ day^{-1}$ with temperatures 20 to 32 °C, respectively. Based on these growth rates, it is possible that if veligers survived being cultured at 24 to 32 °C and 45 ppt over a long period of time, metamorphic competence could be achieved in approximately 40 to 50 days after hatching. High salinity and temperature conditions are known to cause developmental stress due to reduction of dissolved oxygen (Kinne 1964). Therefore, growth and survival of veligers to metamorphic competence could be inhibited in locations where evaporation processes are highest with elevated temperatures.

Future studies need to determine what effects short- and long-term exposure to fluctuations in temperature and salinity have on growth and survival rates of veligers of *S. gigas* at different developmental stages. Kinne (1963) suggested, for example, that a constant temperature of 20 °C and temperatures fluctuating between 15 and 25 °C with an average of 20 °C do not necessarily have the same biological effects. Veligers of *Crepidula fornicata* (Linnaeus) grown under cyclic temperature regimes showed immediate changes in shell growth and carbon content in relationship to each cyclic change in temperature (Lucas and Costlow 1979). Additional studies should also focus on how temperature and salinity may change the documented metamorphic competence criteria. For instance, a recent investigation showed that heat shock (35–37 °C) may induce metamorphosis at a younger developmental stage when no green pigmentation is present (A. Boettcher pers. comm.).

In summary, temperature appears to be the ecological parameter controlling onset and completion of the typical 6- to 8-month spawning season and the geographical distribution of *S. gigas*. Therefore, it is likely that this species has adapted egg production to correspond with the most favorable environmental conditions for larval growth and survival. Egg laying, larval abundance, and temperature are highest during the peak reproductive months, July to September (Stoner et al. 1992). Therefore, recruitment success in these months should be highest based on high number of veligers in the plankton, and the probability that veliger predation decreases with increasing growth and developmental rate (Rumrill 1990). At the beginning and end of the reproductive period larval abundance is low due to lower number of egg masses (Davis et al. 1984, Stoner et al. 1992) and temperatures are below optima for growth and survival. These veligers are likely to disperse to distant populations due to an increase in length of larval life, but settlement success may decrease due to longer exposure to predators and advection from settlement sites (Rumrill 1990). The advantage of variations in larval growth and development during the reproductive season is that this benthic species disperses and recruits to both local and distant settlement habitats which in turn maintains genetic continuity over a wide geographic range.

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DISTRIBUTION AND ABUNDANCE OF *STROMBUS GIGAS* VELIGERS AT SIX FISHING SITES ON BANCO CHINCHORRO, QUINTANA ROO, MEXICO

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ABSTRACT In order to study the distribution and abundance of *Strombus gigas* veligers, duplicate plankton samples were collected every 2 mo from August 1997 to July 1998 at six sites on Banco Chinchorro. Plankton tows were made with a conical net that had a 0.5-m diameter opening and 202μm mesh size. During the sample period, 798 veligers were collected. Larvae were more abundant during the rainy season (July through August), (58.62%, 467 larvae) and "nortes" season (October through December) (35.46%, 283 larvae), while only 5.76% (48 larvae) were counted in the dry season (March through May). Larval density varied from 0.00093 veligers · 10 m^{-3} in May to 7.42 veligers · 10 m^{-3} in August. A high percentage of larvae were stage I (89.08%), with lower abundance of stage II, III, and IV veligers (3.76%, 0.25%, and 6.52%, respectively) and only 0.38% of the larvae were competent. High abundance of early stages suggests that Banco Chinchorro is an important source of veligers. Considering that surface current trajectory in this region is northwestward, the presence of competent larvae in the Southern part suggests an origin in areas outside of Banco Chinchorro, or in places situated downstream. It is possible that Banco Chinchorro supplies larvae to its shelf, the Quintana Roo coast and Florida.

KEY WORDS: Banco Chinchorro, Caribbean, distribution, larvae, queen conch, *Strombus gigas*

INTRODUCTION

The queen conch, *Strombus gigas* (Linné 1758), is a gastropod widely distributed in the Caribbean (Stoner 1997). It has been fished since Arawak Indians inhabited the Caribbean region (Randall 1964, Keegan 1992).

Queen conch stocks have declined throughout the region over the past 10 y, and various regulations have been implemented independently in most Caribbean nations (Berg and Olsen 1989, Appeldoorn 1994). International trade of conch is now monitored by the Convention on International Trade of Endangered Species (CITES) in order to ensure the species' survival.

The biology and ecology of queen conch is relatively well studied (Randall 1964, Brownell and Stevely 1981, Appeldoorn and Ballantine 1982, Stoner et al. 1996, de Jesús–Navarrete and Oliva–Rivera 1997). However, detailed larval descriptions (Davis et al. 1993) and larval distribution and abundance have been surveyed only recently (Stoner et al. 1992, Posada and Appeldoorn 1994, Stoner and Davis 1997).

In Quintana Roo, Mexico the culture of queen conch (Cruz 1984), larval diets and feeding behavior (Aldana–Aranda and Patiño–Suárez 1998), growth of juveniles in pens (de Jesús–Navarrete et al. 1994) and juvenile and adult ecology (de Jesús–Navarrete and Oliva–Rivera 1997), have been studied. Fishery biology investigations have determined that this resource is overexploited in Banco Chinchorro (Chávez and Arreguín 1994).

Recruitment in commercial species with a planktonic phase is complex and is further complicated by the fact that larvae may drift hundreds of kilometers from their site of origin before settling to the benthos. As a result, many local populations depend on distant sources for larvae. Thus, stock management of the species is a multinational problem (Berg and Olsen 1989).

This research represents the first investigation of queen conch larvae in the western Caribbean. The study was designed to determine the abundance and distribution of *S. gigas* veligers and to test the hypothesis that Banco Chinchorro is an important larval production site.

MATERIALS AND METHODS

Study Area

Banco Chinchorro is a false atoll situated offshore from southern Quintana Roo within the Mexican Exclusive Economic Zone (18°23′–18°47′N, 87°14′–87°27′W) (Fig. 1). The bank is geologically similar to the Belize reefs, Turneffe, Glovers and Lighthouse (Jordán and Martín 1987). Chinchorro is 46 km long, 19 km in the widest part, and has an area of 561 km^2. Depth inside the lagoon reef decreases from 12 m in the south region to 7 to 3 m in the central part to 2 m in the north. Chinchorro has four keys, two small keys known as Cayo Norte, Cayo Centro, which is the largest, and Cayo Lobos, the most southerly and smallest. Surface current pattern in the reef lagoon is poorly known. The principal transport is towards the northwest, and the current arrives to Chinchorro from the south.

Sampling

Samples were collected every 2 mo, from August 1997 to July 1998, at six sites in the reef lagoon: Cayo Lobos (18°23′N, 87°23′W), Isla Che (18°29′N, 87°26′W), Cayo Centro (18°35′N, 87°21′W), Cayo Centro West (18°36′N, 87°21′W), Penelope (18°42′N, 87°15′W), and Cayo Norte (18°46′N, 87°20′W) (Fig.1). The dry season is from March to June, and the rainy season is from July to October. The cold season, characterized by strong winds from the north, known locally as "nortes," is from November to February.

Duplicate surface plankton tows were made at each site using

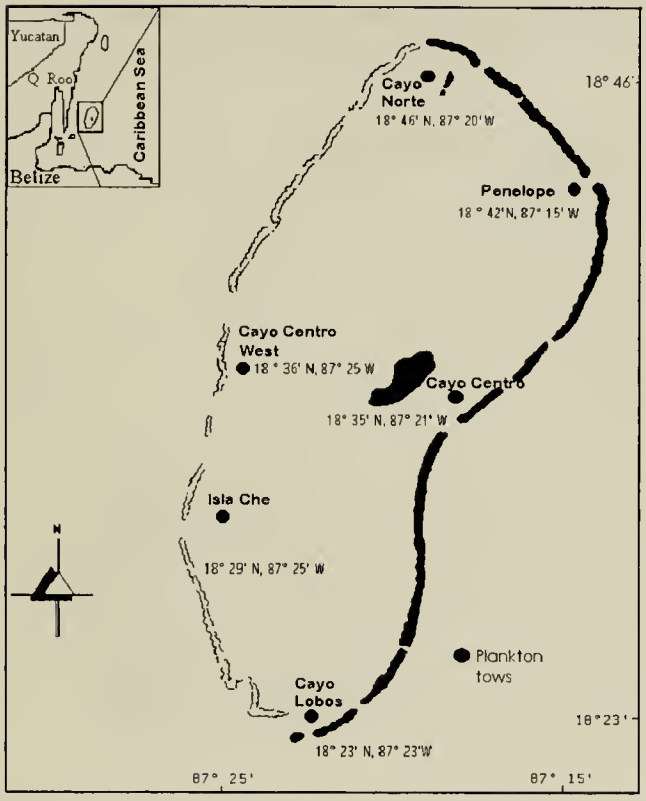

Figure 1. Map of Banco Chinchorro showing the area of sampling locations within the reef lagoon.

a conical net, that had a 0.50-m diameter opening and 202-μm mesh size. Tows were made from a boat traveling in circles, approximately 200 m in diameter, for 15 min at a mean velocity of 1 $m \cdot s^{-1}$. The tow volume was measured using a 2035 MK4 flowmeter display connected to a calibrated General Oceanic™ 2031HR2 flowmeter suspended in the mouth of the net. Plankton samples were preserved in a 5% neutral formaldehyde-seawater mixture (Stoner and Davis 1997). In all sites, tows were diurnal and additional night collections were made only at Cayo Centro and Cayo Lobos.

In the laboratory, the entire volume of each plankton sample was sorted for *Strombus* veligers using a dissecting microscope (20×). Positive identifications for *S. gigas* were made following the descriptions of Davis et al. (1993). Veligers were counted and shells were measured for total length with a calibrated ocular micrometer. Veliger density was standardized to 10 m^{-3}. Larvae were divided into four size classes for analysis of abundance patterns: stage I (150 to 450 μm shell length (SL), Stage II, (451 to 650 μm SL), stage III, (651 to 950 μm SL), stage IV (950 to 1200 μm SL) and competent larvae >1200 μm SL) (Davis et al. 1993). Abundance data were analyzed for spatial and temporal variation using a two-way ANOVA. Data were transformed to Log (x+1) prior to analysis to improve homogeneity of variance.

Temperature (°C) and dissolved oxygen (mg/L) were recorded simultaneously, at the water surface at each site using an oxygen meter (YSI model 58). Salinity (‰) was measured with a temperature-conductivity meter (OHAUS model 50).

RESULTS

Physical Measurements

Temperature ranged from 26.3 ± 0.9 (n = 6) in December to 29.3 ± 0.3 (n = 6) in October. Dissolved oxygen varied between 5.9 ± 0.37 mg/L in July to 7.0 ± 0.56 mg/L (n = 6) in August. Salinity varied from 35.9 ± 0.12‰ in October to 37.0 ± 0.9‰ (n = 6) in March (Table 1).

Distribution and Larval Abundance by Site

A total of 798 *Strombus gigas* veligers were collected from August 1997 to July 1998. Most were collected at Penelope (376 larvae; 47.1%). Cayo Centro followed in importance with 41.0%, which included both the day samples (30.9%) and night samples (10.1%). The remainder were collected at Cayo Norte (4.3%), Cayo Centro West (3.8%), Isla Che (2.9%), and Cayo Lobos (0.9%).

Considering a 4-mo long period for each climatic season, larvae were distributed in the following manner: 58.5% of the veligers were captured in rainy season (July to October), 35.4% was collected in "nortes" (November to February), and 6.0% were collected in the dry season (March to June). On the whole the rainy season and the season of "nortes" contributed 94.0% of the veligers collected.

Larval Density

Density varied from 0.00093 larvae·10 m^{-3} to 7.42 larvae·10 m^{-3}. The greatest density of larvae occurred at Penelope in August with 7.42 larvae·10 m^{-3}, followed by Cayo Centro with 4.95 larvae·10 m^{-3} in October, Cayo Centro (night) with a density of 1.81 larvae·10 m^{-3} in July and Penelope had 1.05 larvae·10 m^{-3} in May. The remainder of the collection sites had densities less than 1 larvae·10 m^{-3} (Fig. 2). There were no significant differ-

TABLE 1.

Temperature (°C), salinity (‰), and dissolved oxygen (mg/L) at Banco Chinchorro Quintana Roo, Mexico, August 1997 to July 1998.

	Cayo Lobos			Isla Che			Cayo Centro			Centro West			Penélope			Cayo Norte		
Month	T °C	‰	O_2	T °C	‰	O_2	T °C	‰	O_2	T °C	‰	O_2	T °C	‰	O_2	T °C	‰	O_2
August	28.2	36.2	7.7	28.6	36.2	7.5	29.0	36.4	6.5	29.8	36.2	6.3	29.3	36.4	6.8	29.0	36.4	7.3
October	29.6	35.8	6.7	29.3	36.1	6.2	29.5	35.9	6.6	28.9	36.1	6.3	29.3	35.9	6.8	29.7	35.9	7.3
December	25.2	36.0	6.1	26.9	36.0	5.8	27.1	37.6	6.5	25.0	36.2	6.2	27.0	36.0	6.0	26.5	36.0	6.2
March	25.9	37.6	6.3	27.1	37.5	6.5	27.5	36	6.2	27.2	37.8	6.4	27.5	37.8	6.0	27.6	37.7	6.3
May	27.6	35.0	6.5	28.2	36.5	6.3	28.4	35.0	6.8	28.4	36.0	5.9	26.5	36.5	6.2	26.5	35.6	6.6
July	29.1	36.2	5.8	29.8	36.2	5.5	29.3	36.4	6.4	29.8	36.0	5.8	—	—	—	—	—	—

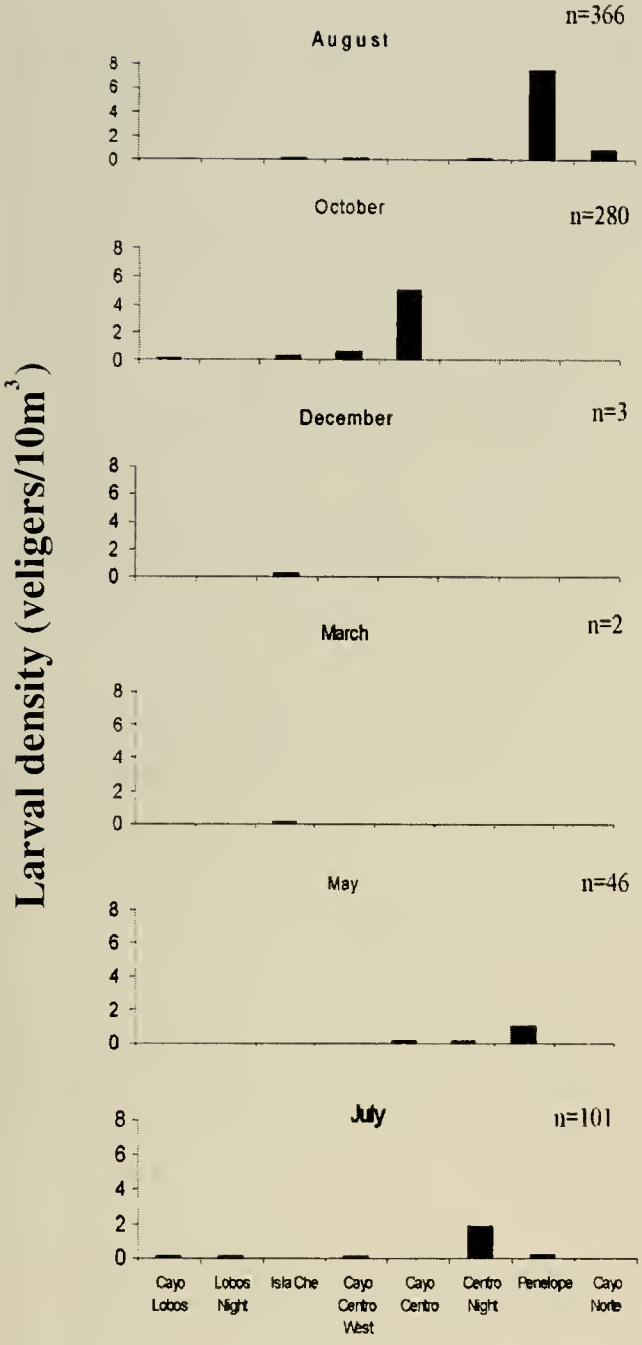

Figure 2. ***S. gigas*** **larval density in Banco Chinchorro by month and site.**

ences in the abundance of veligers between sites or months (two-way ANOVA, Table 2).

Larval Distribution for Size Class

Stage I larvae were most abundant (89.1%, 711 larvae) and 30 larvae (3.8%) were stage II. These sizes were distributed over all sample sites, but with greatest abundance at Penelope and Cayo Centro. Two larvae (0.2%) were stage III (701–950 μm), and were collected at Isla Che and Cayo Centro. There were 52 stage IV larvae (6.5%); these were more abundant in Cayo Centro and

TABLE 2.

Results of two-way ANOVAs for veligers abundance of *S. gigas*, August 1997 to July 1998.

Source of Variation	Sum of Squares	d.f.	Mean Square	F-ratio	Significance Level
Site	14.114	7	2.016	0.943	0.486
Month	19.382	5	3.876	1.814	0.135
Residual	74.797	35	2.137		
Total	108.294	47			

Site refers to sites of sample in Banco Chinchorro.

Penelope. Finally, three competent larvae (0.4%) were collected at Cayo Lobos (Fig. 3).

DISCUSSION

Despite abundant evidence that temperature influences reproduction in *S. gigas* (Randall 1964, Weil and Laughlin 1984, Stoner et al. 1996), Corral and Ogawa (1987) noted that reproduction occurs year round in Banco Chinchorro regardless of temperature. Our results also support the occurrence of year-round reproduction

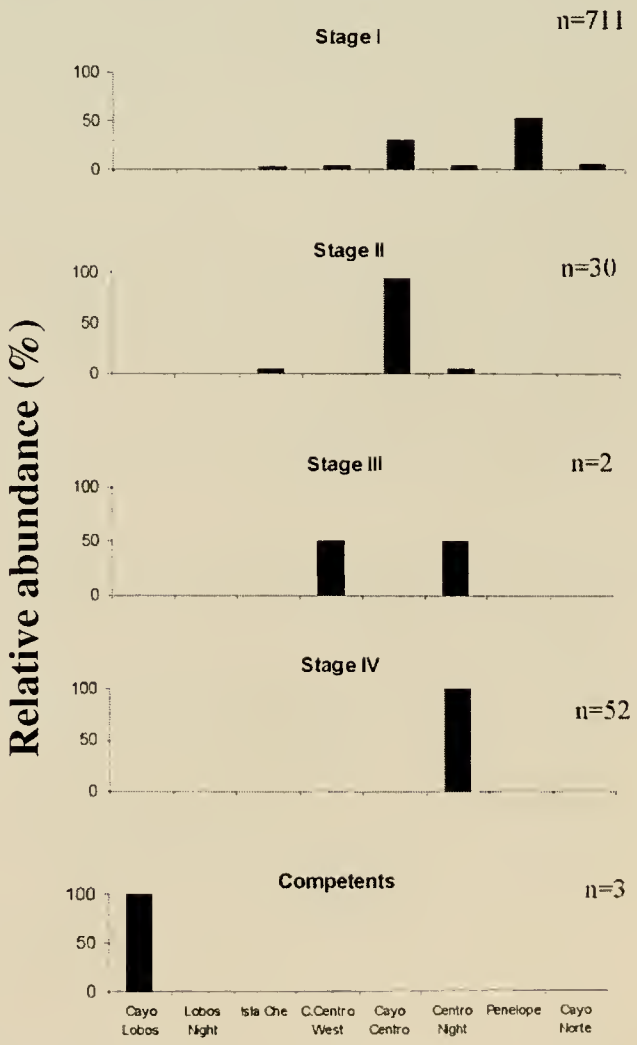

Figure 3. ***S. gigas*** **larval size class distribution in Banco Chinchorro.**

because larvae were collected throughout the year. However, larval abundance peaked in August and October, during the warmer months. Stoner et al. (1992) reported high *Strombus* veliger abundances in the Bahamas during the warmer season (June to September) and Posada and Appeldoorn (1994) also found greater abundance of larvae in July, during the period of reproductive activity at Los Roques National Park, Venezuela.

In Chinchorro, larvae were more abundant at Penelope and Cayo Centro. These sites have tidal channels between the inner lagoon and offshore reef, where the water flow is intense. It is possible that larvae spawned in deep waters, can be transported to reef lagoon by the tidal flow, or action of internal bores, as has been reported for other invertebrate species (Pineda 1995, Shanks 1998, Stoner and Smith 1998).

The larval density found at Chinchorro (0.00093 to 7.42 larvae·10 m^{-3}) is very similar to densities reported in other parts of the Caribbean. In the Bahamas, a maximal density of 4.16 larvae·10 m^{-3} and a minimal of .04 larvae·10 m^{-3} was reported (Stoner et al. 1992). In the eastern Caribbean veligers density in oceanic waters was low (0.20 ± 0.251 larvae·10 m^{-3}) compared with protected zones (0.51 ± 0.45 larvae·10 m^{-3}). The maximal abundance occurred in waters off Los Roques National Park, Venezuela, with a density of 1.22 larvae·10 m^{-3} (Posada and Appeldoorn 1994). In Florida the larval density varied 0.36 to 0.91 larvae·10 m^{-3}. The greatest abundance was found in June and was related with temperature and wind variations (Stoner et al. 1996). The presence of a greater quantity of larvae near competence size than newly hatched larvae (<500 μm) was attributed to a process of transport of larvae from Mexico, Belize or Cuba, and to mesoscale oceanographic processes like eddies in the current from Florida (Stoner et al. 1996).

In Chinchorro, we found a high percentage (89%) of larvae of early stages (I and II; 244 to 780 μm) and this indicates that Chinchorro is an important source of veligers. Stages III and IV stages were not abundant, but they were present, which suggest a continuous local recruitment. In the Bahamas, the presence of intermediate size to 900 μm shell length suggested total development in the Bahamas Bank (Stoner et al. 1996).

There was a variation in the size of larvae from south to north in Banco Chinchorro: the few competent larvae were collected at Cayo Lobos and the vast majority of newly hatched larvae were found in Cayo Centro and Penelope. The presence of competent larvae in Lobos Key, in the south of Chinchorro, indicates the arrival of larvae from the exterior of the bank and possibly from other parts of the Caribbean. Speeds of 0.8 $m \cdot s^{-1}$ to 1.2 $m \cdot s^{-1}$ (Kinder 1983), would permit larvae to cross distances of approximately 900 km between Chinchorro and the eastern Caribbean. Drifters released in Jamaica have passed near Chinchorro and were picked up on the coast of Quintana Roo (Metcalf et al. 1977, Grant and Wyatt 1980).

Even though Chinchorro has a diminished abundance of adults, larval density was high (7.42 larvae·10 m^{-3}). This may reinforce the perception that healthy population of adults is associated with high larval abundance (Stoner and Davis 1997).

It is thought that the Caribbean insular arches located down stream are important sources of larvae within the scheme of metapopulations (Stoner 1997). Banco Chinchorro, is definitively not a source site, since Chinchorro receives competent larvae from the down stream populations and is able to produce larvae from its own shelf. It is likely that larvae disperse from Chinchorro to sites of the Quintana Roo coast and possibly to Florida, since it has been demonstrated that drift cards liberated in Chinchorro arrived in Florida (Merino–Ibarra 1986).

It is possible that Mexico, and particularly Chinchorro, does not receive larvae from Belize, due to the coastal circulation pattern (Merino–Ibarra 1986), and the fact that larvae were not found in Hol-Chan Marine Reserve (de Jesús–Navarrete, unpubl. data). Furthermore, larvae density in the south coast of Quintana Roo was low (1.4 larvae·10 m^{-3}) (Oliva–Rivera and de Jesús–Navarrete, in press). Larvae were present year round in Chinchorro, a characteristic shared only by Florida, and this represents the longest reproductive season in the Caribbean.

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RAPANA VENOSA (VALENCIENNES, 1846) (MOLLUSCA: MURICIDAE): A NEW GASTROPOD IN SOUTH ATLANTIC WATERS

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ABSTRACT *Rapana venosa* (Valenciennes 1846) (Gastropoda: Muricidae), a mollusk native to Eastern Asia, is reported for the first time in Argentine waters in the north of Bahía Samborombón. During a routine bottom sampling, a female specimen of 97.1 and 76.3 mm shell length and width, respectively, and egg capsules were found approximately on 35.3°S-56.4°W in 13 m of water. The possible way of entrance is discussed. The finding of egg capsules permits the supposition that the introduced population is sexually mature and actively breeding. The presence of banks of mussels (*Mytilus edulis platensis*) and oysters (*Ostrea puelchana*), probable prey, together with the occurrence of the egg capsules point out that the development of *R. venosa* in Argentina could be ecologically and economically important.

KEY WORDS: *Rapana venosa*, Muricidae, South Atlantic, mollusca, invasions, Argentina

INTRODUCTION

Rapana venosa (Valenciennes 1846) is a marine gastropod native of Eastern Asia where it is used as a food resource (Hasegawa 1996). Harding and Mann (1999) mentioned the Sea of Japan, the Yellow Sea, the East China Sea, and the Gulf of Bohai as the precise places of origin. Since the description of this species in 1846 it was reported in several countries in Europe and Asia, sometimes as *R. thomasiana*, which is mostly used as a synonym. Powell (1972) recorded pagurized shells of *R. venosa* in New Zealand waters. However, these shells were considered as a food item thrown off an Asian fishing boat (Marshall and Crosby 1998). Drapchin (1953) points out the Black Sea as the first place of penetration out of *Rapana's* traditional geographic distribution. Cesari and Mizzan (1993) mentioned several authors who reported the expansion of this gastropod along the Mediterranean Sea. In 1998, *R. venosa* was recorded in the Chesapeake Bay, U.S.A. (Harding and Mann 1999). This was the first mention of the species in America. Scarabino recorded the same species in Uruguayan waters in April to May of 1998 (pers. comm.).

In this paper we follow the systematic arrangement proposed by Kool (1993). After a phylogenetic study of the family Muricidae he concluded that the genus *Rapana* belongs to the family Muricidae and to the subfamily Rapaninae.

After a routine bottom sampling off Bahía Samborombón, Buenos Aires province, Argentina we found egg-capsules and one adult specimen that belong to *R. venosa*. This constitutes the first written mention of the species in South America.

RESULTS AND DISCUSSION

Egg Capsules and Eggs

Egg capsules of *R. venosa* were collected November 18, 1999 in 13 m of water with a bottom trawl of 120 mm mesh size from 35.436°S-56.373°W (trawl 74, INIDEP EH-09-99) (Figure 1). The whole egg mass (Figure 2) has 208 capsules, which is within the average number (115–220) cited by D'Assaro (1991). Each capsule has an average number of eggs per capsule of 840, ranging from 790 to 890 (n = 20). The egg capsules contained embryos at a morula-gastrula stage, 240 microns in diameter. Collected capsules measured 20 to 30 mm in length, including the curved tip, and 3.5 mm in width at the smallest diameter at the base. The general form and measurements are in agreement with Chung *et al.* (1993) and the detailed illustrations shown by D'Assaro (1991).

Figure 1. Map showing the collection localities of *R. venosa* (Valenciennes) and the egg capsules (✪).

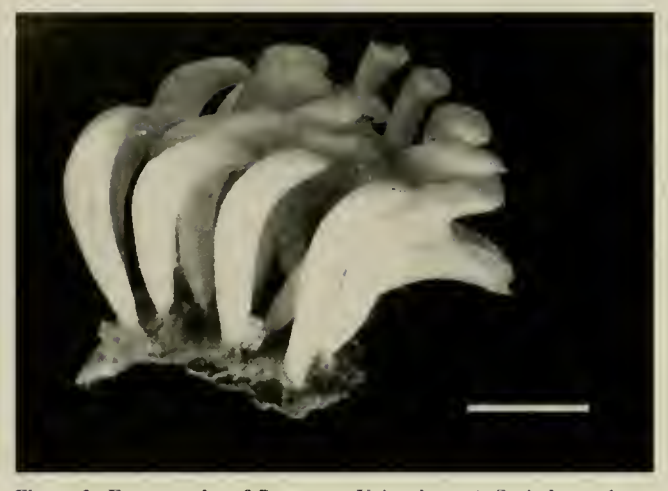

Figure 2. Egg capsules of *R. venosa* (Valenciennes). Scale bar = 1 cm.

Adult Specimen

One female specimen of *R. venosa* (Figure 3, A–C) was also collected on November 18, 1999 in 13 m of water with a bottom trawl from 35.531°S-56.532°W (INIDEP trawl 75 EH-09-99). The specimen was large, reaching 97.1 mm in length and 76.3 mm in width. It has epibiosis of polychaetes Serpulidae and the Cirripedia *Balanus venustus* Darwin. Bottom sediments on both trawls were composed of fine sand.

Together with *Corbicula fluminea* (Müller), *C. largillierti* (Philippi) (Ituarte 1981, Ituarte 1994), and *Limnoperna fortunei* (Dunker) (Pastorino *et al.* 1993; Darrigran and Pastorino 1995) this is the forth species of recently invading mollusks into Argentine waters. However, it is the first gastropod and the first from typical marine environment. All the other invading species of mollusks (all bivalves) are freshwater (*Corbicula* spp.) or eurihaline species (*Limnoperna fortunei*).

Both species of *Corbicula* were apparently introduced as food for crew consumption on Asiatic ships. *R. venosa* is a common and esteemed delicacy in Japan which is called "Akanishi" (Hasegawa 1996). However, this way of entrance seems less probable. Because *Limnoperna fortunei* specimens are not used as food in their native countries it is supposed that the introduction was produced as larvae carried in untreated ballast water from commercial or military ships (Pastorino *et al.* 1993, Darrigran and Pastorino 1995). This is probably the same way that *R. venosa* used to enter North America (Harding and Mann 1999) and Argentine waters as well.

In several papers Carlton (1992 and refs. therein) reviewed probable mechanisms of introduction of non-indigenous marine organisms to North American waters. He mentioned the movement of oysters and the concomitant movement of organisms on the oyster shells or in associated sediments and detritus as one of the most important of these mechanisms. *Ceratostoma inornatum* (Recluz) (Muricidae) was introduced in U.S.A. apparently because of the commercial oyster industry. Another species, *Urosalpinx cinerea* (Say) (Muricidae), was introduced to the northeast Pacific in the same way.

The finding of egg capsules allows us to think that the introduced population is sexually mature and actively breeding. Mussel banks of *Mytilus edulis platensis* d'Orbigny and the local oyster *Ostrea puelchana* d'Orbigny are distributed all along the Argentine northern coast at depth of 35 to 45 m and both are suitable prey for *R. venosa*. The presence of these possible prey together with the occurrence of the egg capsules points out the development of *R. venosa* in Argentina as ecologically and economically important.

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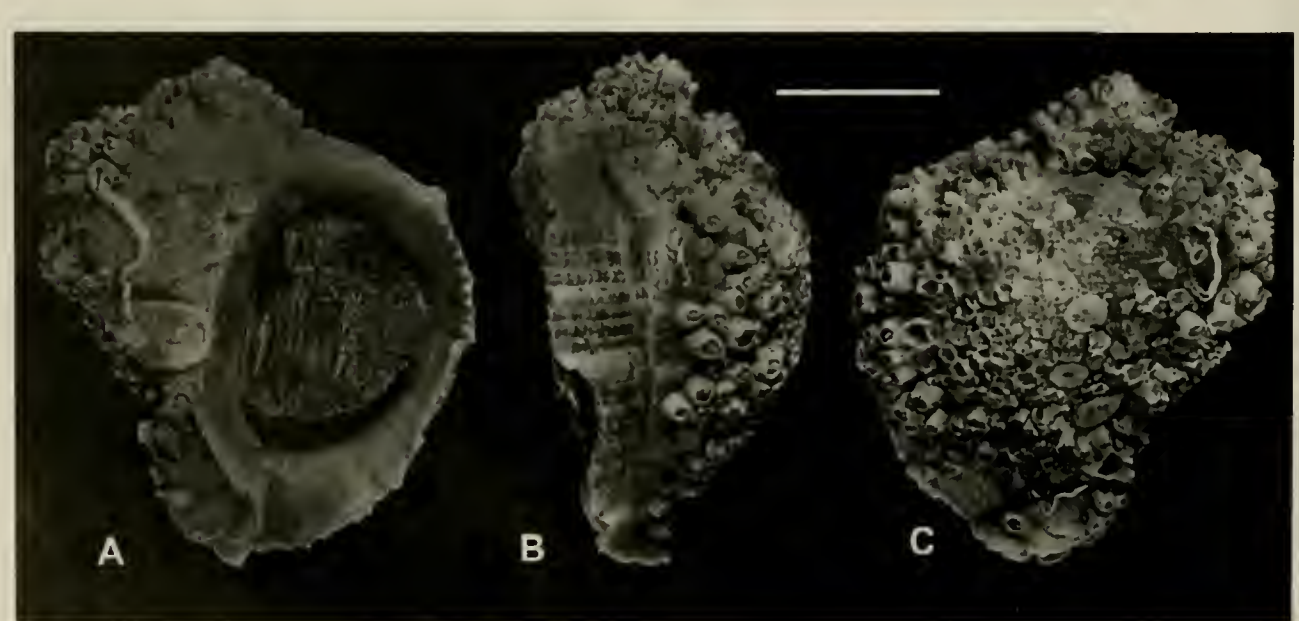

Figure 3. *R. venosa* (Valenciennes). Three views of the female individual collected (A–C). Scale bar = 3 cm.

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SHOREBIRD FEEDING ON STRANDED GIANT GASTROPOD EGG CAPSULES OF *ADELOMELON BRASILIANA* (VOLUTIDAE) IN COASTAL ARGENTINA

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ABSTRACT We report predation by shorebirds on large egg capsules of the gastropod *Adelomelon brasiliana*. This gastropod is a common inhabitant of sandy substrates in the northern coastal area of Argentina and is the only South American volutid known to have free, unattached egg capsules. These capsules are commonly found strewn on beaches, especially after storms. Two shorebirds were observed feeding on embryos inside capsules, the American oystercatcher (*Haematopus palliatus*) and the South American stilt (*Himantopus melanurus*). During periods when egg capsules were stranded both birds switched to prey on embryos inside capsules stranded on the sandy beach. Field observations and a field experiment showed that birds prefer to prey on capsules that have embryos in advanced developmental stages. It is interesting to note that of all the shorebirds observed at the study site, only the local non-migratory species preyed on capsules.

KEY WORDS: Gasteropods, egg capsules, predation, shorebirds, southwest Atlantic

INTRODUCTION

The large marine gastropod *Adelomelon brasiliana* (Lamarck 1811) is a common inhabitant of sandy substrates (5–10 m in depth) in the Province of Buenos Aires, Argentina (36°S to 41°S). This is the only South American volutid known to have free, unattached egg capsules (Penchaszadeh et al. 2000). The first known description of its spawn was provided by d'Orbigny (1846) who recorded large number of egg capsules stranded on the beach in San Blas Bay (40°00′S, 62°30′W). The capsules are oblate-spheroid in shape, yellowish, but nearly transparent, thin with a smooth polished surface like wet gelatin, and possess considerable rigidity (Dall 1889). They measure 40 to 80 mm maximum diameter with a volume up to 140 mL (Penchaszadeh and De Mahieu 1976). Newly laid eggs have an average diameter of 240 microns. Nine to 33 embryos per capsule (Penchaszadeh and De Mahieu 1976) develop, ingesting proteins, amino acids, and sugars contained in the intracapsular liquid and in the inner wall of the capsule (De Mahieu et al. 1974). Capsules do not carry nurse eggs (Penchaszadeh and De Mahieu 1976).

These capsules are commonly found stranded on beaches along the northern Argentinean coast, especially after storms. Eggs and embryos can remain alive on the beach for a period of at least 7 days in winter (P. Penchaszadeh pers. obs.). Once in the intertidal it is unlikely that capsules return to the sea and remain intact because wave motion is too heavy and they would be readily broken. Death of the embryos is mainly from desiccation and increases in temperature. Field observations suggest that some shorebird species utilize this food source.

The southwest Atlantic coastal and estuarine environments, including the coast of the Buenos Aires province are inhabited by several resident shorebirds (i.e. American Oystercatcher *Haematopus palliatus* and South American Stilt *Himantopus melanurus*) and are important stopover and wintering sites for several North American migratory shorebirds (Botto et al. 1998, Iribarne and Martinez 1999). Frequent beach strandings of gastropod egg capsules have been reported in this area (Penchaszadeh and De Mahieu 1976) and shorebirds have been observed feeding on them (O. Iribarne and F. Botto pers. obs.) Given that they may provide a previously unidentified food source, our purpose was to document their use by birds and describe any preference for capsules with embryos in different stages of development.

MATERIAL AND METHODS

The study was conducted near the mouth of one of the easternmost tidal channels (Arroyo San Clemente; 36°22′S, 56°45′W) of Samborombom Bay (a coastal basin 100 km long within the La Plata River estuary, Argentina). The area is characterized by large and dense populations of the fiddler crab *Uca uruguayensis* and the burrowing crab *Chasmagnathus granulata* (Boschi 1964). The littoral zone extends into a large *Spartina*-dominated salt marsh (Bortolus and Iribarne 1999).

During two periods of stranded capsules (December 2, 1998 and January 17, 1999) we sampled the shoreline to obtain an estimate of the density of capsules accumulated per meter of shore. One hundred random samples of 1-m transect were established from the high tide line to the water. Then all capsules were counted in this area at high tide (the width varies from a few centimeters up to 2 m). Given that the intertidal has a variable width, we will report density as numbers of capsules per meter of coastline.

Three study periods (spring through autumn of 1995 and 1996, 1996 and 1997, and 1997 and 1998) were used to generate information on the interaction between birds and capsules. During these periods, monthly or bimonthly field observations were taken in the area observing bird species present and species that fed on egg capsules. A beach (500 m long and 60 m wide at low tide) with

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large patches of *Uca* was used to evaluate bird activity in relation to egg capsules. Observations of bird behavior were performed from mid-morning to late afternoon, and bird species were identified following the field guide of Narosky and Yzurieta (1987). Observers hiding in two different sites recorded bird behavior using 10 × 50 binoculars and an 18 to 36× spotting scope (usually at less than 40 m from the bird activity arena), and data were recorded with a portable tape recorder. Each individual bird was observed for a maximum of 15 min.

To determine if shorebirds prey differentially on capsules with embryos in different stages of development we used two different methods, a comparative field sampling experiment and a field experiment, described as follows in the next two sections.

Comparison of Stages of Embryonic Development of Egg Capsules Stranded on the Beach and from the Subtidal Area

To evaluate possible food preference we compared stranded capsules versus capsules collected by a trawl boat (5–10 m depth) from nearshore areas and classified them into four categories of embryonic development: category I includes embryos not visible to the naked eye, category II includes small, newly formed snails < 5 mm, category III includes embryos 5 to 8 mm in length, and category IV includes pigmented, fully developed snails with calcified shells of about 8 to 11 mm in length. The assumption was that transport of capsules to the beach does not interfere with the embryo's stage of development. We also added two categories of capsules: Pecked which are those capsules that were empty with a clear mark of stabbing by birds, and Broken which are those capsules empty with some kind of rupture, but not clearly identified as bird stabbing. These two categories were added to evaluate the incidence of bird predation on gastropod embryos. A relatively large number of capsules was found stranded on the beach on December 2, 1998, probably produced the previous day. Thus these capsules had been exposed to predation presumably for one day. One hundred-eight randomly (obtained by randomly allocating a 1-m^2 quadrate) collected capsules were obtained to identify the proportion of capsules with embryos at different developmental stages. A sample of 210 capsules was also obtained from the nearshore 5 to 8 m in depth the same day, and embryonic developmental stages were recorded. The null hypothesis of no difference between the frequency distribution of capsules with embryos in different stages, between sites, was evaluated using Kolmogorov-Smirnov test (Zar 1984).

Determination of Feeding Preferences of Shorebirds on Egg Capsules

To evaluate if shorebirds prefer to feed on capsules with embryos at different developmental stages, egg capsules were collected by a trawling boat 5 to 10 m in water depth off the coast of Mar del Plata (38°S, 58°W, Argentina) and classified into the same categories as above. Then 400 of these egg capsules, 100 of each developmental stage, were distributed in the middle intertidal area during a period of low tide. They were left alive stranded on the sand for 4 h during which time shorebirds were observed feeding on them. After this period we counted the number of egg capsules of each developmental stage that were eaten by shorebirds. Then, an index of food selection Chesson's alpha (Chesson 1978) was used to evaluate preference on these food items. The index measures an invariant degree of preference on the part of the predator (Pearre 1982). The index is: Chesson's alpha = $(r_i/p_i) / \Sigma_i (r_i/p_i)$, where r_i and p_i are the proportion of prey item i in the diet and the environment, respectively (Strauss 1979).

RESULTS AND DISCUSSION

During the three study periods we spent a total of 45 days making field observations and we observed seven major strandings of egg capsules. During most strandings the entire field sampling area was covered by a band of capsules approximately 1 m in width. In two of these strandings we sampled the density of capsules and obtained similar values. In the first stranding, density of capsules per meter of coastline was 12 capsules m^{-1} (SD = 21, n = 100), while it was 21 capsules m^{-1} (SD = 16, n = 100) during the second stranding. This amount represents between six and ten thousand capsules accumulated in our study area (500 m of coastline). We have no information on density of capsules stranded during other events, but our impression is that they were similar in magnitude. Given a range of nine to 33 embryos per capsule and a wet weight of 0.15 g per embryo (0.0125 g dry weight), a predator could obtain a total of 1.35 to 4.95 g wet weight (0.1125–0.4125 g dry weight) of food per capsule. This is a significant amount of food that can be readily utilized.

Several species of shorebirds, terns, and gull species were observed feeding in this area during periods of egg capsule stranding. These species were American golden-plover (*Pluvialis dominica*; observed for 260 min), black-bellied plover (*P. squatarola*; observed for 320 min), ruddy turnstone (*Arenaria interpres*; observed for 180 min), whimbrels (*Numenius phaeopus*; observed for 140 min), gull billed tern (*Sterna nilotica*; which dived picking up crabs from the intertidal; observed for 67 min), Brown Hooded Gull (*Larus maculipennis*; observed for 45 min), and the two-banded plover (*Charadrius falklandicus*; observed for 210 min). Most of them (*P. dominica, P. squatarola, A. interpres*, and *N. phaeopus*) are long range (from Canada and the U.S.A. to central-southern Argentina) migratory shorebirds (e.g., Myers and Myers 1979, Morrison and Ross 1989) and in this area, they were found always preying on the fiddler crab *Uca uruguayensis* (see Iribarne and Martinez 1999). The two-banded Plover migrates from southern Patagonia (Argentina) to the northern Argentinean coast during winter (Myers and Myers 1979). None of these species were observed preying on egg capsules. The kelp gull *Larus dominicanus* was seen attempting to feed on capsules (three different days, five individuals). Capsules were picked up in the bill and dropped on the beach from several meters in height. However, this method was not successful, the capsules were never broken, and kelp gulls were not seen eating them.

Two other species of birds were observed feeding in this area, the American oystercatcher (*Haematopus palliatus*; observed for 197 min) and the South American stilt (*Himantopus melanurus*; observed for 235 min). In this area oystercatchers spent most of the time preying on the stout razor clam *Tagelus plebeius*, (Iribarne et al. 1998), while the South American stilt fed on small items including small gastropods (*Littoridina australis*) and newly recruited crabs (F. Botto pers. obs.). However, during periods of capsule stranding they switched to prey on the embryos inside stranded capsules that were lying on the sandy beach. Shorebirds walked along the beach inspecting capsules, strongly pecking some of them to break the capsule and eat the developing gastro-

pods found inside. Pecking by Oystercatchers left a triangular rupture in the capsule wall approximately 20 mm on each side folded into the capsule, while the third side of the triangle remained attached to the wall.

We have no evidence that birds used the walls of the capsule or the liquid inside as food, even though this would be nutritive (Miloslavich 1996, De Mahieu et al. 1974). All evidence suggests that they fed only on the embryos. Egg capsules of gastropods are structurally and chemically complex and very difficult to digest (Miloslavich 1996). Indeed, they can remain on the shore for a long time without degradation. They seem to have strong protection against bacteria, predation, and physical stress, which may be an evolutionary response for egg protection (Pechenik 1986).

Observations on the oystercatcher and the South American stilt behavior were performed while they were feeding exclusively on capsules. Capture of small developing snails from inside the capsule was identified by swallowing action observed in the throat of the bird. Focal sampling of 13 American oystercatchers was performed for a total of 64 min while they were feeding on capsules. The feeding strategy of this shorebird was to "stab" the capsule with its bill, perforating it and introducing their long bill into the capsule. Once inside they probed for snails. These shorebirds needed 43 sec (SD = 23, n = 84) to empty one capsule, eating on average nine embryos per capsule (SD = 5, n = 84). A focal sampling of nine South American stilts was performed for a total of 31 min while they were feeding on capsules. The feeding strategy was similar to the one seen for oystercatchers. These shorebirds needed 25 sec (SD = 18, n = 38) to stop eating in one capsule, eating on average five embryos per capsule (SD = 6, n = 38).

Comparison between the abundance of different stages of development of embryos in stranded capsules (observed after the predation event) with those from subtidal samples showed a distinct pattern. While most subtidal capsules had embryos in an advanced developmental stage, the ones left stranded on the seashore were mainly at the first developmental stages (Kolmogorov-Smirnov test: $P < 0.05$) with a large proportion of eggs broken; most of them were clearly identified as being pecked by birds (see Fig. 1A). The field experiment showed that this difference might be due to differential shorebird predation (Fig. 1B). Only the American oystercatcher was seen feeding in this area during the experiment and the Chesson Index clearly shows they preferred capsules with embryos in advanced developmental stage (III and IV) (Fig. 1B).

Embryos in early developmental stages are very small; they cannot be seen through the capsule walls and are clustered in the lower section of the capsule. However, capsules with embryos in advanced developmental stages (III and IV) are transparent, embryos are larger, (more than 5 mm in length), and in stage IV, they have well-developed feet and usually are seen crawling on the interior side of the capsule wall (Penchaszadeh et al. 2000). These embryos can easily be seen from the exterior through the capsule wall, which may allow visual predators such as the American Oystercatcher and the South American Stilt to select their prey.

There is abundant literature showing predation on eggs by shorebirds (i.e. Crossin and Huber 1970, Farraway et al. 1986). However, we know of no other previous report of predation on egg capsules of prosobranchs. Moreover, of all shorebirds observed in this study, the only two species that took advantage of these capsules were both local non-migratory species (Narosky and Di Giacomo 1993, Martinez and Bachmann 1997, Bachmann and Martinez 2000). The other local non-migratory species was the kelp gull, which attempted to feed on capsules without success. There is no evidence that the two-banded Plover, a short-range migratory shorebird (Narosky and Yzurieta 1987), or any of the long-range migratory shorebirds used capsules as food. Even species like the ruddy turnstone, which feed on eggs of Terns at other sites (Crossin and Huber 1970, Farraway et al. 1986) were not seen feeding on capsules. This pattern may be the result of a food source that is spatially restricted, highly sporadic, and unpredictable, which make them prone to be used by resident species.

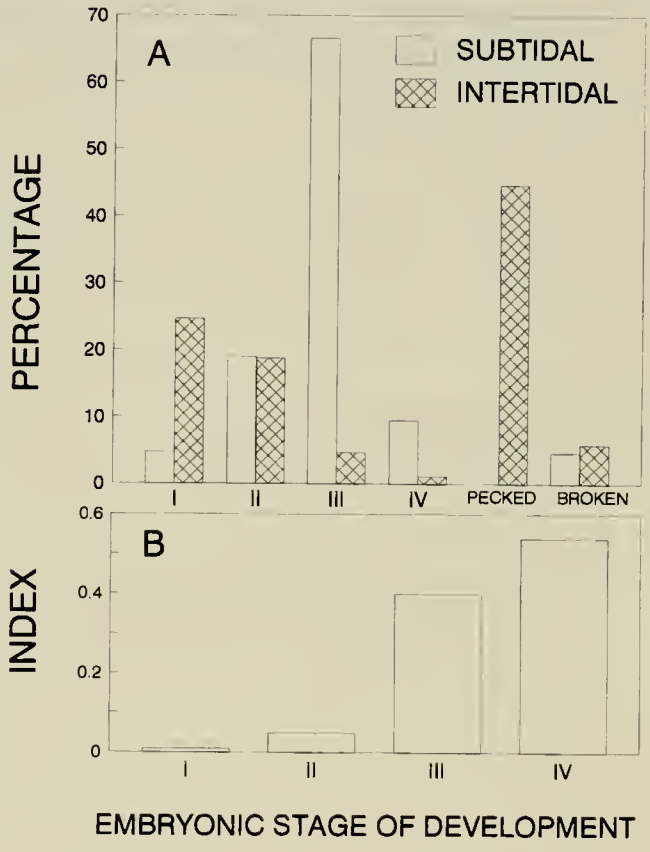

Figure 1. (A) Proportion of embryonic stages found inside egg capsules of *Adelomelon brasiliana* at intertidal and subtidal levels of Samborombom Bay in summer 1998. The categories also include the proportion of damaged egg capsules (BROKEN) and those that had clear marks of bird pecking (PECKED). (B) Cheeson Index of food selection by shorebirds for egg capsules containing different embryonic stages resulting from the intertidal predation experiment.

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NEURAL EXTRACT INDUCTION OF EGG-LAYING AND SUBSEQUENT EMBRYOLOGICAL DEVELOPMENT IN HARD AND SOFT EGG CAPSULES OF THE MARINE SNAIL, *CHORUS GIGANTEUS*

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ABSTRACT The great abundance, long evolutionary history, and diversity of gastropods make this class of mollusks interesting for studies of the evolution and mechanisms of reproductive adaptations. This study investigated induction of laying of egg capsules and subsequent intracapsular embryonic development of the Chilean muricid snail *Chorus giganteus*, whose natural population has suffered a serious decline during the last 20 years. Central nervous system (CNS) extracts (supernatants of homogenates of combined circumesophageal, pedal, and buccal ganglia that were boiled and centrifuged) caused the laying of both soft bulb-shaped capsules and hard well-formed capsules. Latency between injection and capsule laying was 3–6 h. Neither control injections of vehicle (filtered sea water) nor injection of extracts containing less than half of a CNS per recipient caused laying. All but two of 36 capsules laid in response to extract injection contained eggs. Eggs in soft capsules developed normally initially but were subject to infections; although eggs in some hard capsules showed arrested development, in others development appeared to proceed normally, and veliger larvae developed in one hard capsule that was maintained for 49 days. The capsule walls of induced hard capsules had a similar multilayered microscopic structure to spontaneously laid capsules. Soft capsules had a less compact middle lamina with missing or much less compact outer lamina. These experiments demonstrate the presence of a putative hormone activating egg laying in nervous system extracts of *C. giganteus* and demonstrate that normal intracapsular development can occur in some of the resultant capsules.

KEY WORDS: *Chorus giganteus*, development, egg capsule, egg-laying, muricid snail, neurohormone, reproduction

INTRODUCTION

Declines of natural fisheries of some species have prompted research to enhance natural reproduction or to develop aquaculture. For example, in Chile the population of the economically important muricid snail *Chorus giganteus* Lesson 1829 has recently undergone a large reduction. Despite increasing effort, the catch of *C. giganteus* decreased from 2,800 metric tons in 1980 to less than 100 tons annually in 1995 and 1997 (Pinto–Agüero and Soto 1997, SERNAPESCA 1998). Development of aquaculture of *C. giganteus* is hampered by lack of basic knowledge about this species, including about its reproduction. Spawning of *C. giganteus* in the field has been reported in January, March, April, and September (Jaramillo and Garrido 1990); however, the occurrence of egg capsules in the field varies considerably from year to year, making a secure means of obtaining egg capsules a priority in establishing aquaculture for this species. The present study focuses on methods of regulating the reproduction of *C. giganteus* by means of laboratory control of egg-laying and subsequent intracapsular development, with the eventual goal of applying this knowledge to aquaculture.

Marine gastropods employ a variety of methods for packaging and protecting their eggs (Solem 1991). Mechanisms for releasing gametes vary from broadcast spawning followed by external fertilization, as in most vetigastropods, to more highly evolved patterns, found in caenogastropods, that include internal fertilization with deposition of eggs in specialized capsules (gastropod classification according to Ponder and Lindberg 1997). Among neogastropod species, including muricids such as *C. giganteus*, egg capsules are hardened into definitive shapes by the ventral pedal gland (VPG). As described for several species (*Nassarius*, Ankel 1929, *Concholepas concholepas*, Castilla and Cancino 1976, *Busycon*, Ram 1977, *Eupleura caudata etterae*, Gruber 1982), during oviposition, a soft bulb-shaped capsule containing eggs is passed from the female gonopore through a groove in the side of the foot into the VPG, from which it subsequently emerges as a hardened, well-formed capsule. The walls of the egg capsules usually consist of multiple layers. In muricids, these layers typically include an outer protective lamina, a thick middle lamina composed of multiple fibrous layers, and one or two thin inner laminae encompassing the albumen with the suspended eggs (D'Asaro 1988).

Egg-laying behavior and formation of egg capsules in gastropods is controlled by neuropeptides. Induction of egg laying by nervous system extracts (Kupfermann 1967, Strumwasser et al. 1969, Geraerts and Bohlken 1976, Ram 1977) or the purified or synthetic neurohormone (Chiu et al. 1979, Ebberink et al. 1985, Ram and Ram 1989) has been shown in several species, but not previously in any muricid snail. In *Busycon*, the caenogastropod that previously has been most intensively studied, only egg-less capsules were laid unless multiple injections (every 2–3 h for 24 h) were made (Ram 1977, Ram et al. 1982). The present study investigated induction of capsule-laying by nervous system extracts in *C. giganteus*, the presence of eggs in induced capsules, and the effect of the hardening process on capsule wall structure and protection of embryos.

MATERIALS AND METHODS

Animals

C. giganteus for most experiments had been cultured for approximately one year in perforated plastic trays tied to long floating cables anchored in Bahía Metri, adjacent to the Centro de Acuicultura y Ciencias de Mar (CEACIMA-METRI) de Universidad de Los Lagos in Metri, Chile (41°36′S, 72°42′W). Large (>11 cm shell length) females were used as both donors and recipients of nervous system extracts. Recipients were selected from a small number of animals available from trays in which recently laid egg-capsules were present. For experiments, snails were placed individually in 40 cm × 40 cm × 40 cm plastic tanks with constantly flowing 50-μm filtered sea water at a temperature of 15–17 °C.

Recipients in other experiments (pilot experiments and experiments on dosage and sensitivity to ambient temperature) were female *C. giganteus* that had been cultured by J. Navarro in a laboratory culture system in which animals had been observed to lay egg capsules several days prior to injection experiments. Animals in these experiments were held at 15 °C in small aerated aquaria with no flow-through sea water. Experiments were conducted during February and March of 1998 and 1999.

Nervous System Extracts and Injections

Nervous systems were dissected from large female *C. giganteus*. For example, one series of 12 animals had shell lengths of 12.4 ± 0.2 cm (mean ± SEM); wet weight without shell of 60 ± 4 g; gonad weight of 1.5 ± 0.3 g; and gonad index (= gonad weight/wet weight without shell) of 0.023 ± 0.004. Central nervous system (CNS) extracts were made from combined circumesophageal ganglia, pedal ganglia, and buccal ganglia, dissected along with a short piece of esophagus, as described previously for *Busycon* (Ram 1977). CNS and esophagus anatomy of *C. giganteus* is similar to that previously illustrated for the muricid *snail Concholepas concholepas* (Ram et al. 1998). Dissected CNSs were immediately placed on ice and frozen in liquid nitrogen in groups of four. Typically, four frozen CNSs were homogenized in 0.6 mL of ice-cold filtered sea water in a motor-driven glass-teflon homogenizer, then placed on a boiling water bath for 10 min, cooled on ice, centrifuged for 25 min, and diluted to 2 mL to give a concentration of 2 CNS/mL. Extracts were either frozen in liquid nitrogen until needed or injected immediately into recipients.

To test the minimal dosage of extract to elicit egg-laying, an extract containing 2 CNS/mL was diluted 2-fold, 4-fold, 8-fold, and 16-fold in filtered sea water. Diluted extracts were frozen in liquid nitrogen until thawed for injection.

The typical procedure utilized boiled extracts because in *Busycon* the substance eliciting egg laying was known to be stable to boiling but was sensitive to proteolytic or other enzymes in unboiled extracts exposed to ambient temperatures for short periods of time. To test the sensitivity of *C. giganteus* extracts to ambient temperatures, half of an unboiled CNS extract was placed at room temperature for 10 min and then boiled as usual; to the other half of the extract, 0.3 mg benzamil (a protease inhibitor)/mL of extract was added after which the extract was held at room temperature for 10 min followed by boiling. Extracts were frozen in liquid nitrogen until injection into recipients. Animals were injected through the side of the foot with 0.25 mL extract/recipient or, for control injections, 0.25 mL filtered sea water. Similar injections into an isolated foot showed this to be an effective route for injecting into the pedal sinus. The specific timing of injections will be described in the Results. The general sequence of injections was to inject recipients once or twice with extracts, then to inject with control solutions at similar intervals, and finally, to inject again with extracts. Thus, each animal acted as its own control. The rationale for doing two extract injections prior to the control injections in one experiment was that in another species (*Busycon canaliculatum*) multiple injections at a 2- to 3-h interval had been necessary to get insertion of eggs into capsules. Following injection, animals were examined approximately once per hour to look for capsules that had been laid.

Intracapsular Development and Egg Capsule Micromorphology

Egg-containing capsules were placed in aerated vials in filtered sea water at 15 °C, as used previously to study the complete intracapsular development of *C. giganteus* (Gallardo 1981, González and Gallardo 1999). Normally, intracapsular development of *C. giganteus* embryos into veliger larvae and release of the larvae from the capsules takes approximately 70 days at 15 °C (González and Gallardo 1999). The size of the capsules, physical properties and shape (hard or soft, with or without peduncle), and numbers of eggs were noted, and selected capsules were fixed and/or photographed at various developmental stages.

Egg capsules were fixed for 2 h in a mixture containing 2.5% glutaraldehyde, 10% para-formaldehyde, 2% acrolein, and 0.2 M phosphate, pH 7.2 (Rodríguez, 1969). After washing with 0.2 M phosphate buffer, capsules were postfixed for 2 h in buffered 1% OsO_4 and embedded in epon-araldite (Richardson et al. 1960). Semi-thin (1 μm) sections were cut with a glass knife, stained with toluidine blue borax, and mounted for observation under optical microscope. Capsule wall laminae are described with reference to previous studies in other muricacean snails (D'Asaro 1988, Garrido and Gallardo 1993, Rawlings 1995).

Histology

To assess gonadal maturity in both responding and nonresponding females, gonads were dissected, fixed in Hollande Bouin (picric-formol-acetic plus cupric II acetate mixture) (Ganter and Jolles 1970) for 48 h and then dehydrated and embedded using standard procedures. Embedded tissue was sectioned at 6 μm, processed through a series of increasing ethanol solutions, and stained with hematoxylin-eosin (Humason 1962).

RESULTS

Induction of Capsule Laying

Injection of CNS extracts caused the laying of egg capsules. The pattern of responses to CNS extract and control injections in one experiment is illustrated in Figure 1. Five animals were injected twice with CNS extract at an interval of 2.5 h. At the time of the second injection, no capsule had yet been laid; however, approximately 2 h later (a total of 4.5 h after the initial injection), four of the five animals laid an egg capsule. The capsules were soft and bulb-shaped (Fig. 2A), apparently not having entered the VPG. Approximately 3 h later (5 h after the second injection), three of the animals laid another egg capsule, including one hardened capsule attached to the substrate by a typical peduncle (Fig. 2B). All capsules contained eggs, varying from 60–70 eggs in the smallest capsule to 3000 eggs in the capsule shown in Fig. 2B.

TIME (hr)	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
INJECTION #	1			2					3			4			5			6			7				
ANIMAL #																									
1	E			E		$[S]_1$			C			C			E			E			$[S]_5$				$[S]_6$
2	E			E		$[S]_1$			$[S]_2C$			C			E			E			E			$[S]_6$	
3	E			E		$[S]_1$			$[H]_2C$			C			E			E		$[S]_5$					
4	E			E		$[S]_1$			$[S]_2C$			C			E			E			$[H]_5$				$[H]_6$
5	E			E					C			C			E			E							

[ ] = capsule laid; S = soft capsule; H = hard capsule
E = extract injected
C = negative control (filtered sea water) injected

Figure 1. Sequence of injections and capsule laying in response to CNS extracts in *Chorus giganteus*. Time from the beginning of the experiment is shown across the top, and the time of extract injections (E) or control injections (C, filtered sea water) is shown for each animal. [S] or [H] indicates when a soft capsule or a hard capsule was laid, respectively. Subscripts for each capsule indicate the injection that hypothetically caused laying of the capsule. Most extract injections resulted in the laying of a capsule, but some did not. One recipient animal (#5) did not lay at all.

Control injections of filtered seawater into the same animals did not cause laying, demonstrating that it was not simply the effect of injection that caused laying. No capsules were laid within the same time range that previous extract injections had caused laying. When extract was injected again several hours later, animals resumed laying capsules. The fifth animal (i.e., the one that did not lay following extract injections) never laid an egg capsule and died sometime during the next ten days following the above described experiments.

Experiment 2

Ten days after the above experiment, CNS extracts were injected into the four animals that had laid capsules previously in response to extract injections. During the intervening 10 days, no additional capsules were laid; however, all four animals laid a capsule within 4 h of a single injection of CNS extract. At the same time, an additional group of animals was injected with the same extracts. This second group of animals had been maintained at 15 °C in the laboratory but had not previously laid egg capsules. Only the animals that had laid capsules previously responded by laying egg capsules after CNS injections. No animal laid in response to two subsequent sea water control injections; however, three of the four laying animals laid capsules again within 4 h of a second extract injection. One of the responsive animals also laid an egg capsule within the next 10 h during shipment to another location.

Dose–Response Experiment

Three animals that had been maintained by J. Navarro in a closed aquarium system laid capsules when injected with the usual dosage (0.25 mL of 2 CNS/mL extract; i.e., 1/2 CNS per recipient) of *C. giganteus* CNS extract. Subsequently, these animals were injected with 1/32 CNS and successively increasing dosages of CNS extract at 4-h intervals, and observations were made regarding whether or when capsules were laid. Thus, animals were injected with 0.25 mL containing 1/32 CNS, 1/16 CNS, 1/8 CNS, 1/4 CNS, and 1/2 CNS. One animal did not respond at all (and, in fact, did not respond to several subsequent maximal dosage injections). The other two animals laid a capsule only after the highest dose (1/2 CNS), each laying a single soft capsule approximately four hours after the injection.

Ambient Temperature and Benzamil Experiment

Three animals that had laid capsules in response to boiled CNS extracts were injected with an extract that had been incubated at room temperature for 10 min prior to boiling. None of the recipients laid a capsule within 4 h of the injection. Subsequent injection of a benzamil-treated extract elicited laying of a soft capsule from two of the three recipients approximately 3.5 h after the injection.

In addition to the above experiments, capsules were also laid in a pilot experiment following CNS injections into two animals but not following control injections of seawater.

Size of Capsules, Number of Eggs, and Intracapsular Development

Altogether, CNS extracts stimulated 10 animals to lay at least one egg capsule, with 8 of these animals responding at least two or more times. These animals laid a total of 36 egg capsules, including 28 soft capsules and 8 hard capsules. The hard capsules ranged in length from 18 to 23 mm (median = 21 mm), whereas soft capsules were 7 to 20 mm (median = 14 mm) in length. All but two capsules contained eggs. The number of eggs per capsule in six hard capsules in which eggs were counted ranged from 550 to 3,050 (median = 1,700), whereas two of the largest soft capsules

Figure 2. Soft and hard capsules laid by *Chorus giganteus* in response to extract injections. (A) capsule $[S]_1$ of animal #2 (see Fig. 1). (B) capsule $[H]_2$ of animal #3 (see Fig. 1). Calibration: 10 mm.

contained 1,100 and 1,550 eggs. The diameters of the eggs in the hard capsules ranged from 250 to 260 μm (median = 253 μm), whereas the diameters of the eggs in five soft capsules averaged 249, 255, 262, 264, and 323 μm.

Embryonic development occurred in some induced hard capsules, but in several others development appeared to be arrested or slower than normal. Eggs in four capsules maintained for 29 to 49 days before fixation had reached only the polar body stage or had undergone only the first division. However, normal cleavage was observed in eggs of one capsule that was fixed 1 wk after being laid. Another hard capsule, maintained at 15 °C for 49 days, contained several veliger larvae (Fig. 3A), along with more than 900 non-developing eggs. By comparison, another hard capsule that had been laid spontaneously by one animal prior to the beginning of these experiments contained 8 veliger larvae (Fig. 3B) and 1,449 uncleaved eggs after being maintained for approximately 50 days.

Intracapsular development also took place in soft extract-induced capsules. Soft capsules were fragile and more subject to infection than hard capsules. A few of the soft capsules showed tears in their wall, even though handled gently. The eggs in these capsules had a normal appearance. In other soft capsules, embryos underwent apparently normal initial development. Eggs in one soft capsule that was fixed 1 wk after laying had embryos that had achieved a multi-cellular stage, similar to embryos in normal hard capsules. Nevertheless, soft capsules maintained longer than a week all eventually became infected with various pathogens (bacteria, protozoans, nematodes, etc.) within 5 wk. Of eight soft capsules maintained in the laboratory at 15 °C for 23 or more days, six contained uncleaved and/or broken eggs, one exhibited some eggs with polar bodies, and one that was terminated at day 35 due to infection had been observed on day 25 to contain trochophore larvae that had ingested nurse eggs.

Microscopic Structure

Susceptibility to infection and damage could be due to structural differences in the capsule wall. Micrographs of both hard and soft capsules are shown in Figure 4. The wall of a hard capsule laid in response to extracts (Fig. 4A) shows the layered structure of a normal hard capsule (Fig. 4B), including (1) the outer lamina, (2) the middle lamina, and (3) the albumen-retaining lamina. Another layer, the lax lamina, would be situated between laminae 2 and 3, but the lax lamina is only 0.5-μm thick and therefore not visible in these micrographs. The middle lamina is the most complex, including a compact outer layer, a middle porous vacuolated layer, and a compact inner layer. In the normal and extract-induced hard capsules of *C. giganteus*, the middle lamina was approximately 20–25-μm thick. Two soft capsules (Figs. 4C and 4D) had a less-compact middle lamina, with much thicker vacuolated regions and total widths of 36 μm and 45 μm. In addition, the layered structure of one of the soft capsules (Fig. 4C) is incomplete in that the two outermost coverings (both the "outer lamina" of the surface and the underlying "compact outer layer") are practically absent. In the other soft capsule (Fig. 4D), the wall layering was relatively complete, although the outer lamina appears relatively less compact than in a hard capsule.

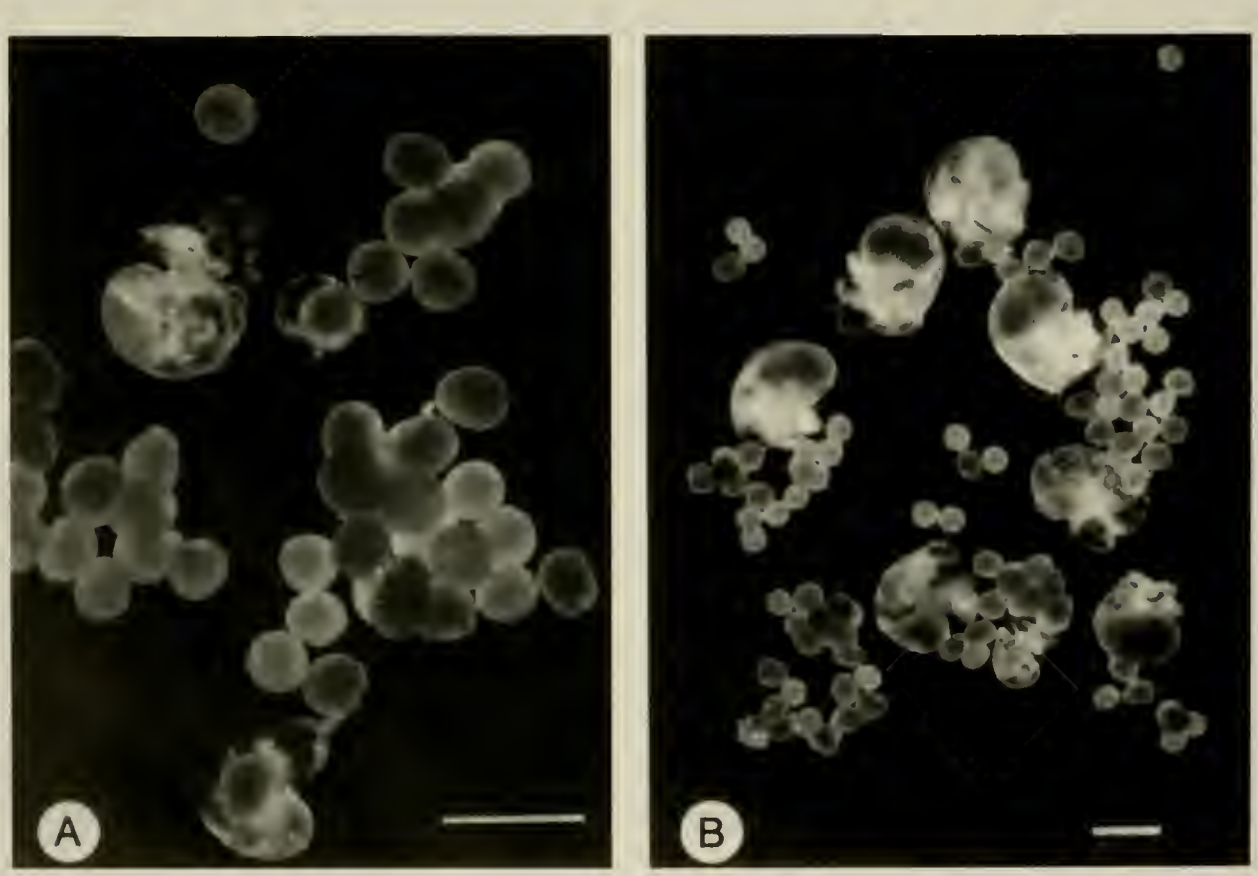

Figure 3. Intracapsular development. (A) Veliger larvae and non-developing eggs present in hard capsule $[H]_5$ (see Fig. 1) when fixed 49 days after being laid. (B) Veliger larvae and non-developing eggs in a hard capsule spontaneously laid by animal #3 prior to injection experiments. Calibrations: 500 μm.

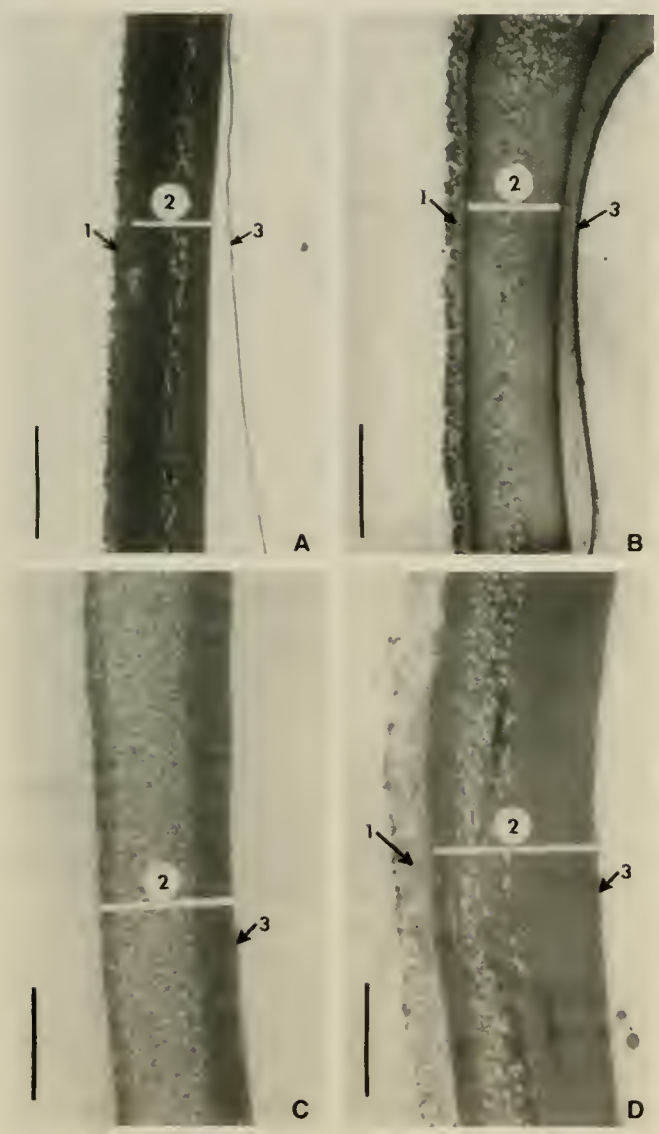

Figure 4. Micrographs of capsule walls from hard and soft capsules: (A) hard capsule $[H]_6$ (see Fig. 1), induced by extract injection. (B) hard capsule laid spontaneously by animal #3 prior to extract injection. (C) soft capsule $[S]_5$ laid by animal #3 in response to extract injection. (D) soft capsule laid by animal #4, in response to extract injection, in experiment conducted 10 days after the experiment illustrated in Fig. 1. Laminae of the capsule walls are labeled as follows: 1, outer lamina; 2, middle lamina; and 3, albumen retaining lamina. Calibrations: 30 μm for all.

Histology of Responsive and Non-Responsive Recipients

Histological studies of the gonads of the four responsive females that laid eggs in response to nervous system extracts showed clearly that three had a ripe ovary (animals 1, 2, and 3 in Fig. 1) while the other was in active maturation (animal number 4 in Fig. 1). Six additional females (previously conditioned for reproduction at the laboratory in Metri) that were not responsive to injections of CNS extracts were also studied to address their gonadic stages; three had ripe gonads and three were in active maturation.

DISCUSSION

This article is the first to demonstrate induction of egg-laying by nervous system extracts in a muricid snail. Eggs were present in the first induced capsules of responding animals and in all but 2 of 36 induced capsules altogether, a result that is different from capsules induced by a similar method in *Busycon*, another neogastropod (Melongenidae). Because *C. giganteus* laid normal hard capsules as well as soft capsules, these experiments enabled us to study the role that the hardening process has on protecting the eggs, which has not previously been accomplished. Our micrographic studies illuminate the structural differences between hard and soft capsules.

Although not every extract injection caused the laying of an egg capsule, the laying that did occur was clearly in response to the extracts. In Experiment 1 (Fig. 1), 17 extract injections resulted in the laying of 13 capsules; in experiment 2, 8 extract injections resulted in at least 7 capsules. Animals never laid capsules after control injections within the same time period as after extract injections. Furthermore, no capsules were laid by animals during the 10 days following the end of experiment 1, showing that spontaneous laying was rare for these animals. In the dose-response experiment, low concentrations of CNS extract failed to elicit laying.

We hypothesize that the egg-laying factor in *C. giganteus* CNS extracts is a heat stable peptide. Retention of activity after boiling is similar to egg-laying neuropeptides in *Aplysia* spp., *Lymnaea* spp. (both Heterobranchia, traditionally classified as Opisthobranchia and Pulmonata, respectively), and in *Busycon* spp. (Caenogastropoda) (Kupfermann 1970, Ram 1977, Ebberink et al. 1985). The egg-laying hormones of *Aplysia* and *Lymnaea* are homologous 36-amino acid peptides known as egg-laying hormone (ELH) and caudo-dorsal cell hormone, respectively (Chiu et al. 1979, Ebberink et al. 1985). In *Busycon*, the inducing agent is protease sensitive (Ram 1977) and approximately the same estimated size by gel filtration as ELH (Ram et al. 1982). We hypothesize that the loss of activity of *C. giganteus* CNS extracts during a 10-min incubation at room temperature prior to boiling is due to endogenous proteases, consistent with preservation of its activity by addition of the protease inhibitor benzamil.

Despite the similarities in size, protease sensitivity, and heat stability of the *Busycon* egg-laying factor to *Aplysia* ELH, they have distinct differences reflecting the divergence of gastropod subclasses 350–400 million years ago (Goodman et al. 1988). Thus, inter-species injection experiments between *Aplysia* (Heterobranchia) and *Busycon* (Caenogastropoda) do not cause laying in the other species (Ram et al. 1982). Determination of the primary structure of the active factors causing laying in caenogastropods would help identify the structural differences that account for the lack of inter-class activity. Demonstration of the egg-laying activity of CNS extracts in *C. giganteus* suggests that such studies could also be pursued in this species.

The latency of the egg-laying response to CNS extract injection was generally longer *in C. giganteus* than in other gastropods. In experiment 2, in which each *C. giganteus* was injected with extract only once prior to observing laying of an egg capsule, the median latency was 4 h. The shortest latency observed in any experiment after single injections was about 3 h. The data on latency in Figure 1 are difficult to interpret, since two extract injections were given prior to the laying of the first capsule. However, by hypothesizing that each injection elicited the laying of no more than one capsule [as previously observed in *Busycon* (Ram et al. 1982)], the latencies between CNS injection and laying are estimated to vary from 3 to 6 h. In comparison, latencies to lay after hormone injection were 30 min in *Aplysia* (Stuart et al. 1980, Strumwasser 1984,

Ferguson et al. 1989), 100 min in *Lymnaea* (Dogterom and van Loenhout 1983, Geraerts et al. 1988), and 2–4 h in *Busycon* (Ram 1977).

The number and presence of eggs in most induced capsules of *C. giganteus* differs from *Busycon*. In *Busycon*, the initial 5–15 capsules laid in spontaneous egg-laying episodes are devoid of eggs (Ram et al. 1982). Subsequently laid capsules average approximately 35 eggs per capsule (Ram et al. 1982). A single extract injection into *Busycon* generally elicits only one egg-less capsule, and repeated injections over a 24-h period are necessary to obtain capsules containing eggs (Ram et al. 1982). In contrast, for *C. giganteus* almost all capsules contain eggs, and the number of eggs per capsule is usually >1000 (Castillo and Ulloa 1998, González and Gallardo 1999). Thus, the presence of eggs in nearly all capsules induced by CNS extract in *C. giganteus* reflects the normal pattern of egg deposition in this animal.

These experiments demonstrated that normal development can take place in induced capsules. Trochophore larvae developed in one soft capsule before development was disrupted by infection, and veliger larvae were obtained in an induced hard capsule. Development of only a small percentage of eggs into larvae is usual for this species, in which a large proportion of eggs serve as nurse eggs to developing embryos, and eggs in many capsules may not develop at all (Castillo and Ulloa 1998, González and Gallardo 1999). In the present study, in one spontaneously laid capsule only eight veliger larvae out of approximately 1,500 eggs developed. Previous studies of *C. giganteus* reported 7–12% of encapsulated eggs developed, the remainder being used as nurse eggs (Castillo and Ulloa 1998, González and Gallardo 1999). Arrested development has also been observed in capsules collected from the field, in which up to 45% of the capsules in a spawn exhibited arrested development (Castillo and Ulloa 1998, González and Gallardo 1999). The number of eggs developing per capsule obviously can vary a great deal in both natural and induced spawn. A challenge for aquacultural development of this species will be to attain an optimal ratio of developing eggs to nurse eggs in every capsule.

Capsules laid by *C. giganteus* in response to CNS extracts were successfully transported to the VPG for hardening in only a minority of cases, providing an opportunity to study the effect of hardening on capsule wall structure and function. Whereas the wall structure of induced hardened capsules was similar to spontaneously laid capsules, the walls of soft capsules were less compact and/or were missing layers. This difference in wall structure of the soft capsules may account for their greater susceptibility to pathogens. Previous authors have suggested that protection of embryos from microorganisms is a major function of gastropod capsule walls (D'Asaro 1988, Garrido and Gallardo 1993, Rawlings 1999) and that capsules from *Nucella emarginata* from which two layers had been mechanically stripped were much more vulnerable to protists and predators (Rawlings 1994, Rawlings 1995). Compaction or addition of layers to the capsule wall by the VPG may impart this biotic protection to the capsule.

Application of this method for inducing the laying of egg capsules to culturing *C. giganteus* may be hindered by the necessity of producing hard capsules and by the difficulty of ascertaining which animals will respond. Our histological studies comparing responsive and non-responsive animals did not reveal any differences in gonadal maturity. The lack of responsiveness of seemingly mature animals to effective stimuli has been noted in other molluscs (Ram 1977, Ram et al. 1993). Determining what additional factors regulate responsiveness to spawning inducers is one of the most critical unsolved problems in understanding and reliably controlling reproduction in these animals. The low numbers of developing embryos in this study may also indicate a limitation in applying these methods to mass-production of snails. Finally, the high dosage of nervous system extract needed to induce laying of egg capsules indicates that this could be an efficient method to obtain more snails only if the inducing substance (presumed to be an egg-laying peptide hormone similar to those sequenced in other gastropods) could be synthesized economically for injection into responsive animals.

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EFFECT OF REPETITIVE DYE EXTRACTION OVER YIELD AND SURVIVAL RATE OF THE PURPLE SNAIL *PLICOPURPURA PANSA*(GOULD, 1853)

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ABSTRACT The purple snail is an important economic species because of the dye obtained from it in western Mexico. This dye has been used since ancient times to color ceremonial dresses purple. Other snails produce dye, but *Plicopurpura pansa* dye extraction was done without killing the snail. Repetitive dye extraction is possible. The best time between each milking, dye yield versus milking frequency, and effect on survival was determined by repetitive milking snail groups several times (7, 14, 21, and 28 days). Mortality in the most frequently milked groups and dye yield reduction occurred (every 7 and 14 days). When milking frequency occurred every 21 days, the best dye yield and 100% survival rate was observed. These results suggest wild populations can be exploited using optimum extraction schedules, leaving at least 21 days between each dye extraction.

KEY WORDS: mollusks, purple snail, exploitation, natural dyes, *Plicopurpura pansa*

INTRODUCTION

Several animal and vegetable products have been used for milennia by different cultures to supply dyes (Baranyovits 1978). Among all ancient natural dyes, those using marine gastropods were the most prestigious, and the textile industry established was one of the most important and complex in Europe and Mideast (Koren 1995). Several species from genera *Purpura, Plicopurpura, Murex,* and *Thais* were used to obtain purple and blue dyes (Baker 1974, Fox 1966, Ghiretti, 1996). These colors have been symbolically related with important and powerful people.

At the intertidal zone of the eastern tropical Pacific, purple snail or dye snail *Plicopurpura pansa* (Gould, 1853; Synonymous: *Purpura pansa*), is a valuable species because of the fluid produced in the hypobranchial gland. After secretion, this dye changes from its initial white color to purple by a chemical reduction. Unlike other snail dye producers in which it is necessary to break the shell and to kill the animal to obtain the dye gland, dye extraction from *P. pansa* is made by mechanically exciting the snail foot and operculum, so multiple milkings can be made. Friedlander determined that 12,000 *Murex* snails were necessary to obtain only 1.4 g of Tyrian purple dye, after a complicated extraction process. This explained the rarity and high cost of such dyes in the past (Friedlander 1908 in Baranyovits 1978).

Natural colors are again becoming desirable, and their use for dying fabric is increasing. In the late 1980s on the rocky shores of the Mexican Pacific coast, a small-scale fishery of purple shell was developed to support a Japanese market for dye of expensive kimonos. However, this fishery was looking for the highest dye yield, and the time between milkings was not considered; therefore, the local purple shell stock was depleted in a short time (Turok et al. 1988). Since 1988, the Mexican Government has had *P. pansa* under special protection (Anonymous 1988, Anonymous 1994). Currently, the purple snail is used only by the Mixteco people on the coast of Oaxaca to make ceremonial dresses. This activity has existed from before the time of Columbus (Turok 1996).

To study reproduction (Acevedo 1995) and feeding aspects (Memije 1994, Montiel 1993, Rentería 1996), purple snails were examined under controlled conditions. Ríos-Jara et al. (1994) used tagged wild purple snails to determine dye yield and recovery time after being milked. They found a relationship between each of these variables and the shell length, but the effect of repetitive dye extraction was not established. González (1996), through a complex experimental design, tried to establish the best time between milking; however, his results are not clear.

The aim of this study was to establish the minimum time between milking without detriment to snail survival. A 3-month experiment with several snail groups and repetitive milking at different times was carried out. The results can be used for the assessment and planning of potential exploitation of this species.

METHODS

Purple snails (n = 110) were collected by hand in June 1997 from the intertidal zone on three rocky shores of the Mexican Pacific coast (Fig. 1), on the south side of Isla Socorro, Archipielague Revillagigedo, in Cuastecomates Bay, on the Jalisco State and at Pescadero Point, State of Baja California Sur (BCS). Many snails from different areas were collected to consider the conditions of several habitats and the possible intrinsic variability of its population dynamics.

Snails were transported wet to the Laboratory of Experimental Biology at CICIMAR, in La Paz BCS, Mexico. Two fiberglass 200-L aquariums were used for the experiment. Each aquarium had an opening and semicontinuos sea-water-filtered system. 400-L/h power-head pumps and different size stones were used to simulate natural substratum and splash condition. The water temperature was the same of adjacent sea (23–26 °C), the salinity ranged from 36 to 38 ‰ and a 12-h daylight cycle was established.

Figure. 1. Collect sites of purple snails in Mexican Pacific coast.

In the tidal rocky shores, this species preys upon a variety of other mollusks, invertebrates, and dead animals. In the laboratory, they were fed daily with fresh squid chunks. Several other kinds of food were offered during acclimation period, but squid meat was the preferred one. Water and laboratory temperatures were recorded daily and corrected as required. Full aquarium cleaning was carried out daily.

Before any manipulation, a 2-month acclimation period was spent before the experiment. Five groups with 20 snails of different sizes (shell length range: 19.8–61.6 mm) and both sexes were conformed at random (female:male ratio at field, 1:0.95). All snails were kept together, and each snail was tagged with a particular color group and a plastic number on the conch. Snail size and wet weight were recorded to the nearest 0.1 g through an electronic caliper and digital scale, and the data were entered into a database. Each aquarium had 47 snails, two groups each with 20 snails, and a control group of seven snails. Most of the mortality recorded occurred after collection and during acclimation.

After the acclimation period, the groups were submitted to a series of milking, according to an established schedule. For milking, each snail was taken from the aquarium, excess water was removed with a towel, and the expulsion of the dye was stimulated by blowing and slightly pressing on the foot and the operculum. The dye obtained from each snail was stored individually in Eppendorf vials.

All groups were submitted to an initial milking on 28 August, and the last milking took place on 21 November, 1997. Subsequent to the initial milking, each group was exploited on different dates according to the 3-month period of the experimental design. The control group was not milked (Gw). One group was milked every 7 days (G7), one every 14 days (G14), one every 21 days (G21), and one every 28 days (G28).

At each milking, the dye produced by each snail was collected and the volume measured. To determine the volume of dye, a regression equation between volume and the weight of the dye was made, using the data of initial milkings. Later, the volume produced by each snail was calculated by determining the weight of the vials and using the regression equation. A daily control of the mortality in each group was recorded.

After the 3-month period experiment, the sex of each snail was determined, stimulating manually the exit of the body from the shell and then determining the presence of penis or genital orifice. Although the sex of each of the snails in the experiment was identified, we decided not to consider this variable within the analysis, because secondary sexual dimorphism is not present, and therefore it is not easy to distinguish the sex of the snails collected and milked in a commercially exploited stock.

To determine possible differences in the conformation of different groups, an analysis of variance (ANOVA) and Tukey tests with untransformed data were carried out to compare the shell length and the wet total weight of the snails (Zar 1996). Confidence limits were prefixed at 95%. The mean volume of dye of all snails at initial milking was determined, and the anomalies in dye production for each size group were calculated with reference to the initial value.

To compare dye production under different milking regimes, data on dye production was standardized by dividing the volume produced per snail by the snail length. Normality tests, analyses of variance, and Tukey test were completed to compare dye extraction of each group and among different groups (Zar 1996). The analysis was done using Statistica for Windows 95.

Regression equations were fitted to the data of the shell length and volume of dye per animal, the frequency of milking, and the total dye volume by group. Survival data after milking was described by the differential equation of numbers of survivors against milking times. A polynomial regression was used to describe the relationship between the frequency of milking as a variable depending upon the product of total dye volume and survival.

RESULTS

The mean temperature during the experiment was 24.5 °C in the aquaria. The characteristics of shell length, weight, and sexual proportion of each group of snails is shown in Table 1. The minimum length was 19.8 mm, and the maximum 61.1 mm. The average shell length for all the groups was 35.1 mm. The multiple comparison test does not show significant differences among the different groups ($P > 0.05$), in length or in weight. The sexual proportion observed in the different groups approached a 1:1 male—female ratio.

Figure 2 shows the results of the survival rate observed in each one of the groups subjected to different milking regimes during the study. Groups with dye extraction at 21 and 28 days showed no mortality. In contrast, groups with a milking frequency of every 14 days (G14), had a 95% survival rate, and the beginning of mor-

TABLE I.

Size, weight, and sexual proportion of snail groups under different milking regime.

	Groups					
Parameters	**G7**	**G14**	**G21**	**G28**	**Gw**	**Total**
Average size[a]	35.5	36.0	36.3	34.7	32.4	35.1
Minimum size[a]	25.3	25.0	24.5	27.3	19.8	19.8
Maximum size[a]	53.3	51.8	61.1	51.3	52.5	61.1
SD in size[a]	8.5	7.8	11.2	7.4	10.1	8.9
Average weight[b]	7.8	8.3	9.0	6.9	8.1	8.1
Minimum weight[b]	2.5	2.6	2.8	2.9	1.4	1.4
Maximum weight[b]	21.1	22.3	30.6	16.9	24.2	30.6
SD weight[b]	5.8	6.3	8.9	4.6	7.8	6.7
n females	11	10	9	11	6	47
n males	9	10	11	9	8	47

[a] Length size, mm.
[b] Weight, g.

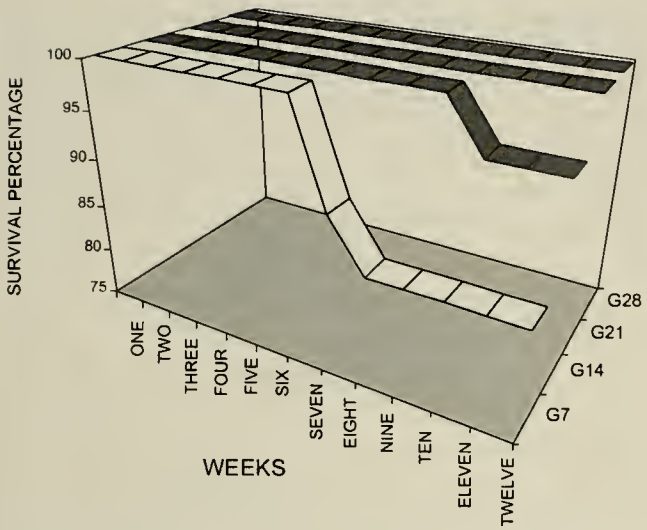

Figure. 2. Percentage of survival for each purple snail group during milking period.

tality was shown in the ninth week (at the fourth milking). In the group (G7) with weekly dye extraction, the lowest values of survival rate (85%) were observed, and snail mortality began at the sixth week (at the fifth milking). An accidental death of a snail in the control group was recorded at the second week of the experiment.

Evidence shows the possible existence of a moderate effect of milking frequency on survival; however, the values of dye production analyzed for each group indicate that repetitive dye extraction is important.

The mean volume of dye produced in relation to shell length is shown in Figure 3. The volume of dye/snail using the data of the first milking was determined, and an average value of 0.47 mL/snail was obtained (SE m 0.03). It was not possible to obtain dye in 12 out of 94 snails (12.7 %). A power regression establishing the relationship between the length (L) and the volume of dye (D) of the snails was established, with a correlation coefficient value of r = 0.64. The equation follows:

$$D = 0.0003*L^{2.10}$$

Figure 4 shows the error (observed value minus the mean) with respect to the average volume of dye for each group of snails at

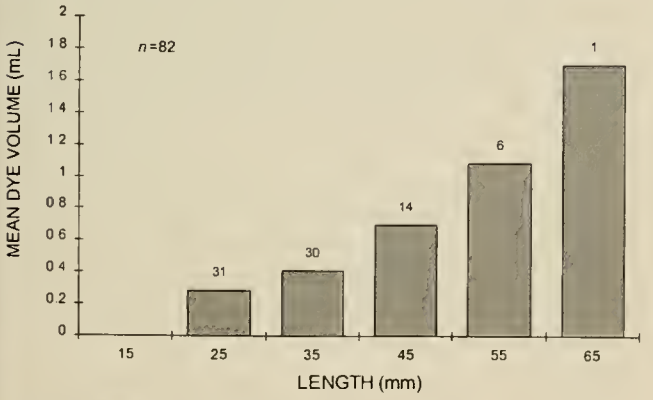

Figure. 3. Mean dye volume and size of each group, for all snails at initial milking.

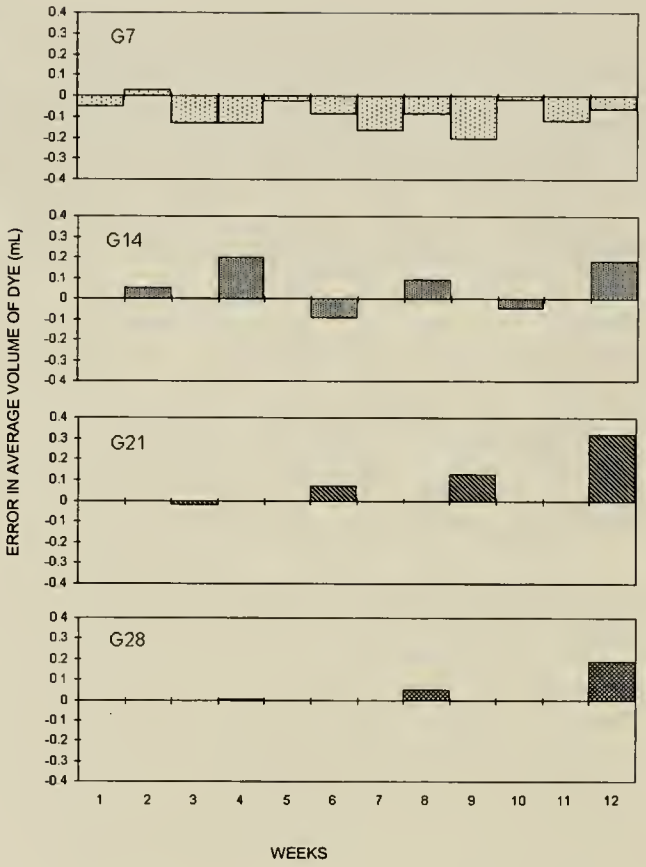

Figure. 4. Errors in average yield for each milking and snail group.

each milking. The dye extraction determines the recovery of dye production. Thus, in group G7, a decrease in the volume of dye produced is observed; in G21 and G28 by contrast, an increase in the average production after the second milking is observed.

There are no significant differences among the different groups at initial milkings (Tukey test, $P > 0.05$), but significant differences at the final milkings (Tukey test, $P > 0.05$) are shown.

If the accumulated total volume obtained in each milking regime is considered, in G7 the maximum dye volume is obtained after the 3-month period. Although individual extraction and milking tend to yield smaller volumes, the higher milking frequency (12 in total) implies a greater accrued volume. Group G28 shows the opposite pattern, high dye yields per milking but a smaller accrued volume (Table 2). This information is relevant if intense management of the purple snail is planned. However, when the dye yields along with the survival rate observed during the study period for each group (Figs. 5a and 5b) are analyzed, the product of dye volume by survival (Fig. 5c), shows the maximum yields would be obtained by milking every 10 days, which assumes the possibility of a certain mortality induced by the handling of these animals by the fishers.

Looking for conservative use wherein the lowest mortality induced by repetitive dye extraction prevails, we conclude the optimum period between each milking is 21 days.

DISCUSSION

The results obtained depart from the implicit assumption of an adequate conformation of experimental groups, because differ-

TABLE 2.

Accumulated dye volume (mL) for each snail group during milking period.

Date	Week	G7	G14	G21	G28	Gw
5 September	1	7.7				
12 September	2	9.5	9.5			
19 September	3	5.8		8.8		
26 September	4	5.8	12.8		8.0	
3 October	5	6.3				
10 October	6	6.6	7.3	9.3		
17 October	7	4.0				
24 October	8	5.8	10.7		9.4	
31 October	9	3.3		10.8		
7 November	10	7.8	7.3			
14 November	11	4.6				
21 November	12	5.4	10.5	13.5	11.9	6.5
Accumulated dye volume		73	58.2	42.4	29.4	6.5

ences in mean length or weight were not observed. Equally, the sexual proportion is about 1:1.

The values observed in this study of dye yield with respect to size are lower with respect to those cited in the literature on purple snails with similar size or weight in the wild (Michel-Morfín et al. in press, Ríos-Jara et al. 1994), but are coincident with those of previous laboratory studies (González, 1996). This difference could have two causes. Dye extraction under controlled conditions allows complete removal of water that is incorporated into dye from the mantle cavity. In the intertidal zone, this is difficult to achieve because of the uncontrolled conditions. This may cause overestimation of dye volume.

In addition, data obtained under experimental conditions can differ from values obtained under natural conditions, because the snails were fed with a monospecific diet, in this case squid chunks. The nutritional condition of experimental snails could vary with respect to those from the intertidal zone, which have diverse prey. This could have an effect on dye production. Even with this possible effect, the influence of milk frequency found should be considered to obtain the optimum dye yield in the field.

The role of dye in snail physiology should be studied in depth under specific experimental designs, even though it has been mentioned to have a feeding role (Bandel 1987 in Kool 1993, Ríos-Jara et al. 1994), during the experimental period there was no evidence recorded for the use of dye for this purpose. We did not detect traces of dye in the food that was withdrawn from the aquaria, but experimental purple snails were fed with processed food; they did not have to catch and crack open their own. Castillo-Rodríguez (1995) mentions two feeding mechanisms, and the dye is possibly used for immobilizing prey. More needs to be discovered about this subject.

A 3-month experimental period was taken under the assumption that over this time the effect of repetitive milkings would be seen. In addition, it has been the minimum time in which the Mixteco tribes from Mexican Pacific coast historically devote to this activity each year (Turok et al. 1988). However, the possibility of carrying out repetitive milks over a longer period could be evaluated to determine the possibility of dye extraction of wild stock for more than 3 months.

The results suggest the possibility of exploiting purple snails in

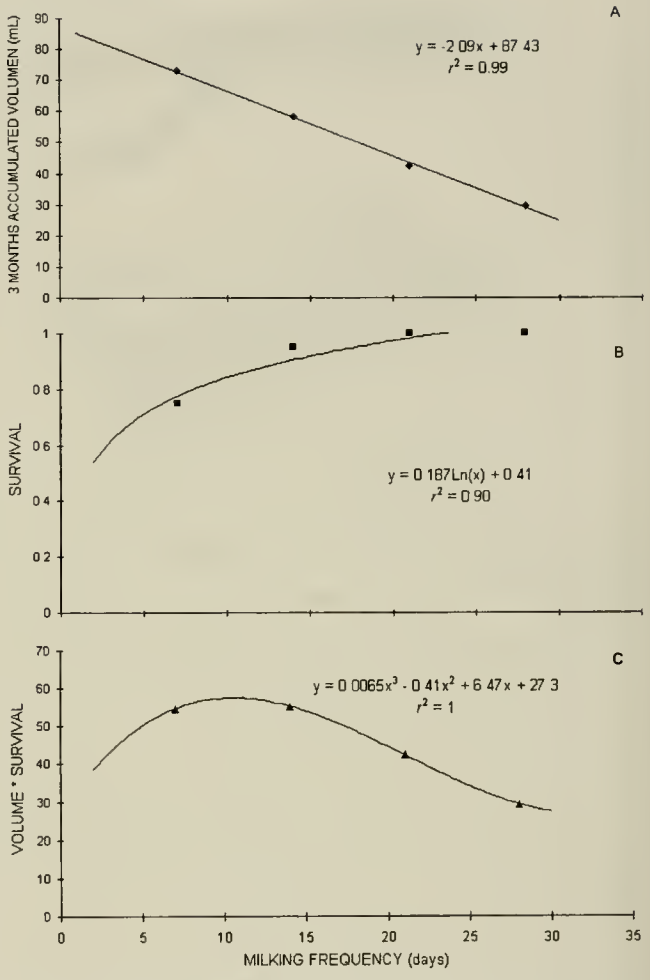

Figure. 5. Relation between A) dye accumulated volume, B) survival, and C) the product of volume-survival with respect to milking frequency.

the wild assuming that, if a minimum period of 21 days between each milking is used, no mortality will be induced by handling the snails. This should be a decisive element for the definition of a management strategy, given the explicit requirement to avoid depletion of any exploited stock. Dye extraction must be done carefully and snails also must be carefully handled to protect them from the wave action and isolation, putting them on the same sites from where they were taken to permit them to attach to the rocks again. The process from the moment each snail is removed, milked, replaced, and reattached to the grounds takes between 5 and 10 minutes. As long as these simple rules are followed in a commercial activity, it is possible to have a low impact on the purple snail.

The Mixteco people have used this resource for direct dying of cotton from ancient times, collecting snails along the Oaxaca coast and leaving 4 weeks between each milking from September to November. There is some evidence that this activity was more intensive in the past and was done without any adverse effect on the populations (Turok et al. 1988, Castillo-Rodríguez and Amezcua-Linares 1992).

Although *P. pansa* is currently under special protection by Mexican law, it is important to do further research to determine the

real potential of the snail exploitation. Commercial use of purple snail could be made on specific zones and under particular conditions using local zones where the stock has high densities and snails are larger. This activity could be profitable to native people. In areas such as the coasts of Jalisco and Colima, no indigenous people make use of this resource. There are some groups re-evaluating the use of natural dyes and interested in the exploitation of this dye with a high added value; therefore, this activity could be reactivated.

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POPULATION PARAMETERS AND DYE YIELD OF THE PURPLE SNAIL *PLICOPURPURA PANSA* (GOULD, 1853) OF WEST CENTRAL MEXICO

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ABSTRACT The purple snail (*Plicopurpura pansa*) is a conspicuous rocky shore species of the intertidal zone of tropical western America. It is considered a potential resource due to the dye it produces. Estimates of population density, sex ratio, growth parameters, mortality, and recruitment were obtained from bimonthly samplings from 1993 through 1995 on a rocky shore of west central Mexico. A different growth rate between sexes was observed. Mortality coefficients for the population are estimated for the first time. Recruitment to rocky shores occurs principally from September through March. The life span (longevity) was estimated as 11 y. Dye production related to size and sex was determined and is described by an exponential relation.

KEY WORDS: *Gastropoda, population parameters,* Plicopurpura pansa, *purple dye, Mexico*

INTRODUCTION

Some species of gastropods mollusks like *Purpura, Plicopurpura*, and *Murex* are remarkable for their ability to produce dye. From ancient times, many cultures have used these inks to dye ceremonial dresses, often associated with religious traditions and power (Baker 1974, Baranyovits 1978, Turok et al. 1988, Clark et al. 1993, Ghiretti 1996).

One of this group of species, commonly known as purple snail or dye snail *Plicopurpura pansa* (Gould 1853, sinonimous: *Purpura pansa*), is a common inhabitant of rocky shores in the intertidal zone of tropical western America (Fig. 1). Its distribution is typical Panamic, ranging from Baja California to southern Colombia and the Galapagos Islands (Keen 1971).

The hipobranchial gland of the purple snail secrets a fluid that turns intense purple on the contact with sunlight and air (Ríos–Jara et al. 1994). In contrast with other dye-producing snails, the dye produced by *P. pansa* is easy to extract without sacrificing the snail, so one can obtain multiple milkings.

In Mexico, the Mixtecos, an indigenous people on the Pacific Coast, use the dye secreted by *P. pansa* combining it with other natural inks such as cochineal carmine, from the pearl cactus insect *Dactylopius coccus*, and indigo, from plants of the genus *Indigofera* (Turok et al. 1988, Turok 1996). The extraction of purple dye is made at the shore by dying a wet cotton mop directly with the dye purple from the snails.

A few previous studies on the yield production of dye as related to size and sex have been performed (Turok et al. 1988, Alvarez 1989, Castillo–Rodríguez and Amezcua–Linares 1992, Holguín, 1993, Ríos–Jara et al. 1994). Among these articles, two have attempted to describe growth rate (Turok et al. 1988, Alvarez 1989). No estimation of natural mortality has been made.

The purple snail can be considered a potential resource because of the dye obtained from it. In the late 1980s, a small-scale dye exploitation to support a Japanese market for expensive kimonos was developed in west Mexico. However, this activity was made without technical and biological regulations, and negative effects on the snail population were evident (Turok et al. 1988).

Some studies on population dynamics and the effects of milking on snail populations are necessary to evaluate the real potential of this activity. During the last few years, there has been an increased interest in natural dyes, probably because some of the artificial ones tend to cause sensitivity and toxicity problems (López 1993).

For this reason, the goal of the present study was to determine population parameters and dye yield as a first step to obtain basic information about this species. This information, together with other work now in progress, can help in determining the viability of a fishery for purple dye and the best way to manage this resource.

METHODS

Bimonthly samples to measure density and length frequency were made from May 1993 to January 1995 (except November 1994) in Bahia de Navidad, Jalisco, México (19°13′29′N and 104°43′45′W, Fig. 2). A 50-m long by 2-m wide transect was established in the intertidal zone along the shoreline. All snails found were recorded, and each snail was milked by blowing and slightly pressing on the foot and the operculum to stimulate the expulsion of dye. The volume of dye obtained from each snail, the length of each shell, wet weight, and sex were recorded. All snails were released at the same site after sampling.

Length was recorded from the apex to the most distal point of the anterior siphon canal (Fig. 1, bottom). Because the purple snail has no sexual dimorphism, the sex of each snail was determined by manually stimulating the exit of the body from the shell and then determining the presence of penis or genital orifice.

The length–weight relation was established by fitting a power regression to the data. Differences in sex ratios were tested by a slope-comparing t test (Zar 1984). Growth was described by the von Bertalanffy growth model (VBGM) with the use of the Fisat

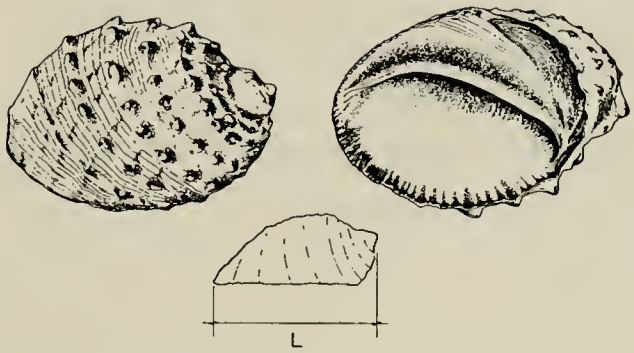

Figure 1. Purple snail *Plicopurpura pansa* dorsal view (top left) ventral view (top right) points of reference to length of shell (bottom center).

software package (Gayanillo et al. 1995) to fit sampling data and estimate recruitment and the fishing mortality coefficient. Estimates of natural mortality coefficient were obtained by using diverse methods described by Sparre and Venema (1992) and Chávez (1995) using a computerized spreadsheet.

The relationship between length and dye volume was determined by power regression. Sex differences was tested by a slope-comparing *t* test (Zar 1984).

RESULTS

A total of 964 snails was sampled in all the study months, 380 females and 288 males. The sex of 296 snails could not be determined. Table 1 shows sexual ratios and abundance by month. The average female:male ratio was 1:0.75. Although sexual proportion favored females in most months, sexual proportion was between 1:1 in March to 1:0.53 in May. The mean overall density was 1.7 snails/m^2 (SD = .34). No relation between density and sexual proportion was found.

The method for sex assessment in the field is not always effective (only 70% of snails were sexed from 964 collected) because it is necessary to sex snails one by one under difficult conditions (rocky terrain with much wave splashing) and it must be done quickly to prevent snail desiccation and death. The method is particularly difficult with large snails, and maybe this situation could affect the sexual proportion values.

Lengths ranged between 7.8 and 79 mm (mean = 32 mm, SD = 12.6) for females and from 9 to 76.4 mm (mean = 28.9 mm, SD = 8.6) for males (Fig. 3). The lowest modal value, at 14–18 mm, corresponds to recruits to the rocky shore. Snails with sizes >50 mm are few and are mainly females.

Estimates of condition factor or α value (.0003 and .0002) and slope value β (2.85 and 2.9) for females and males suggest isometric growth (Fig. 4). A good fitness to length–weight power regression is shown for both sexes (r^2 of 0.94 for females and 0.90 for males). Slopes were significantly different between sexes ($P <$ .02).

The bimonthly length-frequency distributions for each sex, asymptotic length ($L\infty$), and growth coefficient estimate (K) were calculated (Table 2). There is a different growth rate between sexes. Females have a higher annual growth rate (K = .27) and a higher $L\infty$ value (110 mm). These values are consistent with the information obtained from the length-frequency histograms for each sex.

For the estimation of $L\infty$, the Fisat package contains a routine with the Powell and Whetheral method, and another one with the

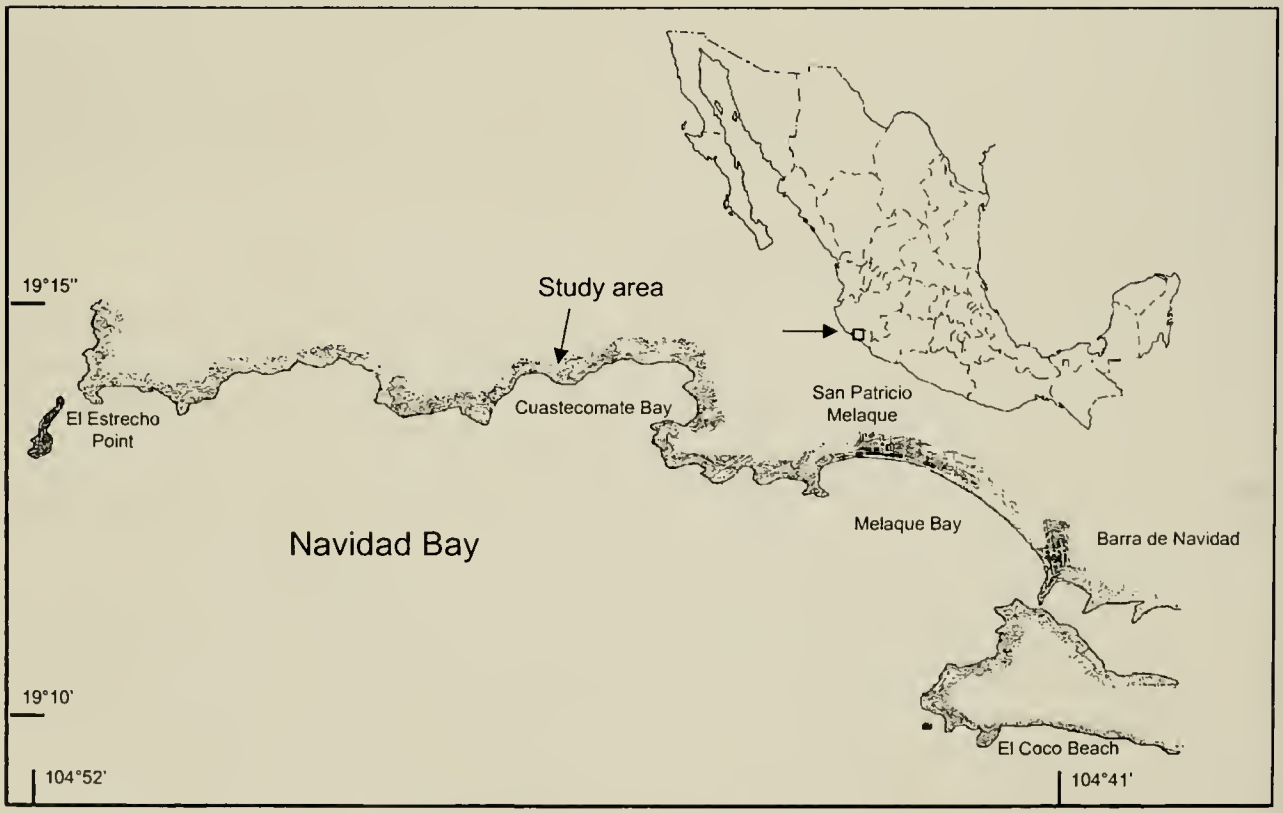

Figure 2. Study area. Bahia de Navidad, Jalisco.

TABLE 1.

Sex ratio and density of Purple snail *Plicopurpura pansa* in the Pacific Coast (May 1993 through January 1995).

Month	Sex Ratio (%)		Density
	Females	Males	Snails/m^2
May 1993	65	35	2.0
July 1993	58	42	1.3
September 1993	57	43	2.0
November 1993	54	46	2.1
January 1994	58	42	2.0
March 1994	50	50	1.5
May 1994	59	41	1.5
July 1994	57	43	1.2
September 1994	55	45	1.3
January 1995	56	44	1.5
Mean	57	43	1.7

Shepherd method and ELEFAN to estimate K values. The former one provided a better estimation (see Gayanilo et al. 1995 for details). In Figure 5, the score function axis shows the best fit of the Shepherd function (Pauly and Arreguín–Sánchez 1995).

Other values were obtained using the Munro and Gulland and Holt methods in the Fisat package and are consistent with our estimates done by tagging methods for both sexes together, where K = .26 and $L\infty$ = 110 (n = 9). Figure 6 shows the goodness of fit of the growth estimates of the von Bertalanffy model (VBGM) for each sex. In addition, through an empirical relation between longevity and growth rate (K), an estimate of life span as 3/K, ranging from 11 to 13 y, was determined.

The snail population of the rocky shore at the study site is not exploited, hence, it is valid to assume that total mortality (Z) is equal to the Natural Mortality (M). For this reason, several methods to get estimates of coefficients M and F were considered and tested. Several mortality estimates are shown in Table 3, ranging from 0.21 (y^{-1}, where K = M, according to Chávez 1995) and 1.47 as given by the catch-curve method, another routine in the Fisat software package. Most estimated values range between 0.21 and 0.47, except those obtained by the Jones and van Zalinge and catch-curve methods.

Our field observations show the reproductive season occurs between January and May and is characterized by snail couplings and clutches of egg capsules in rock crevices. Our own laboratory observations suggest that the time of larval development inside the capsule is about two to three months. Later, a planktotrophic larval stage hatches, whose time length is not known, but it is suspected that it may take about 6 mo.

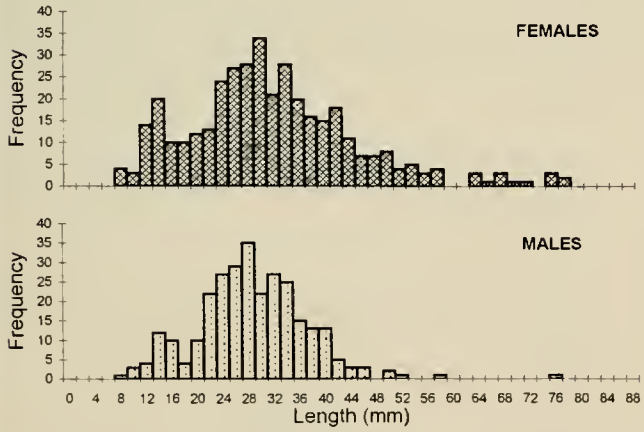

Figure 3. Length–frequency histograms of male and female purple snails.

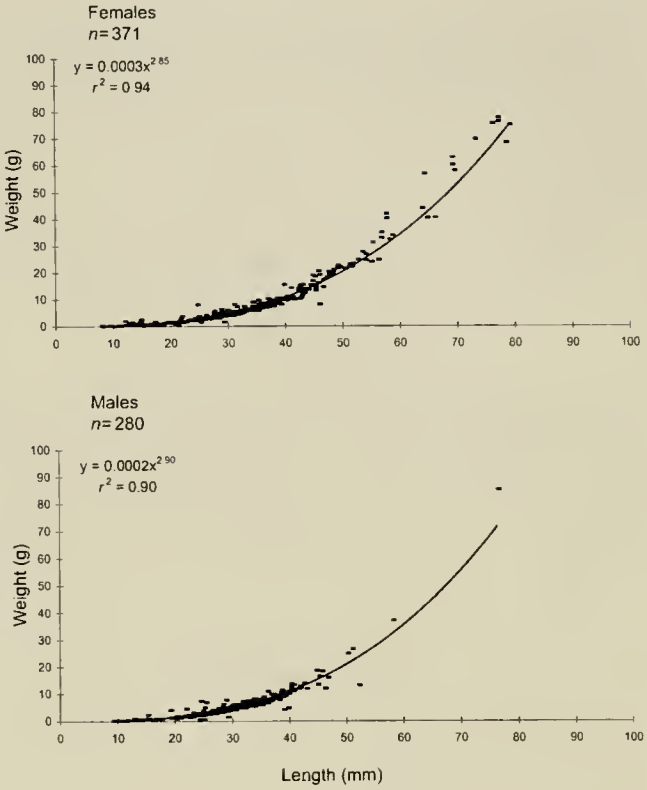

Figure 4. Length–weight relation for male and female purple snail *Plicopurpura pansa* in Bahia de Navidad, Jalisco.

In our data series, recruitment occurs from July 1993 to May 1994. A second period of recruitment was seen from September 1993 to January 1995, but this could start earlier (November 1994 was not sampled, Fig. 7). The minimum size observed was 7.8 mm and the maximum size was 20 mm. The mean length of recruitment to the rocky shores is 15 mm (SD = 3.05). From this data and our growth parameter estimates, age group 1 (recruit size) must be about 1 y after hatching.

Dye yield increased exponentially with length for both males

TABLE 2.

Growth parameters ($L\infty$, K, t_0, $W\infty$), and length-weight parameters (α, β) estimated for purple snail *Plicopurpura pansa* females and males using the Fisat software package.[a]

Attributes (Units)	Female	Male
$L\infty$ (mm)	110	102
K (y^{-1})	.27	.21
$W\infty$ (g)	198	134
t_0	−0.04	−0.04
α	0.0003	0.0002
β	2.85	2.90

[a] See Gayanilo *et al.* (1995) for details.

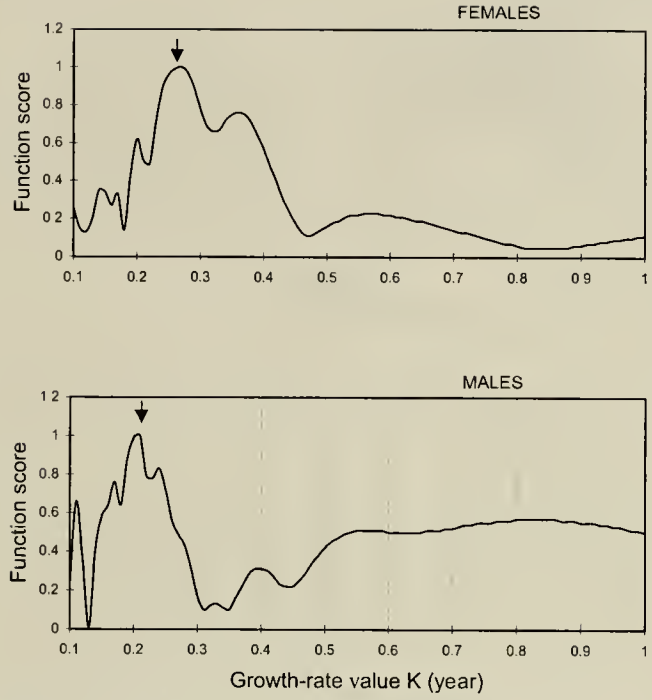

Figure 5. Estimates of growth rate value (K) by Shepherd method for females and males.

and females (Fig. 8, Table 4). The best comparing slopes did not show significant differences between sexes ($P > .1$). However, the large variation in dye produced is evident, especially in large sizes (>50 mm). This variation may be related to the use of dye by the snail. The mean dye yield was 1.88 mL/snail (SD = 1.69) for females, 1.23 mL/snail (SD = .85) for males and 1.81 mL/snail (SD = 1.6) for all the snails sampled (female, male, and unsexed specimens).

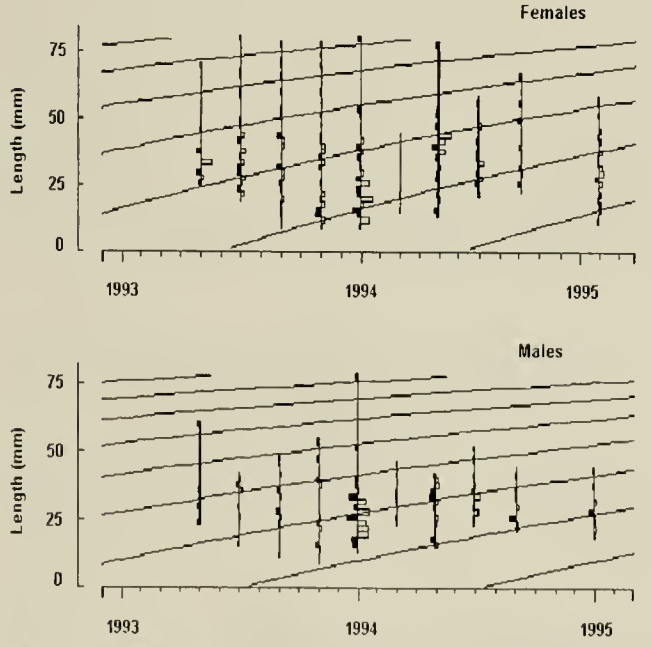

Figure 6. Growth curve of female and male purple snail *Plicopurpura pansa* in Bahia de Navidad, Mexico for years 1993 through 1995.

TABLE 3.

Estimates of natural mortality (M) for purple snail *Plicopurpura pansa* population using several methods.

Method	Estimates of Natural Mortality (M)	
	Females	Males
Alagaraja	.27	.21
Ault and Erhardt	.36	.27
Beverton and Holt	.47	.38
Catch Curve	1.51	1.79
Chávez	.27	.21
Hoenig	.39	.36
Jones and Van Zalinge	1.43	1.45
Rickther and Efanov (modified)	.39	.36

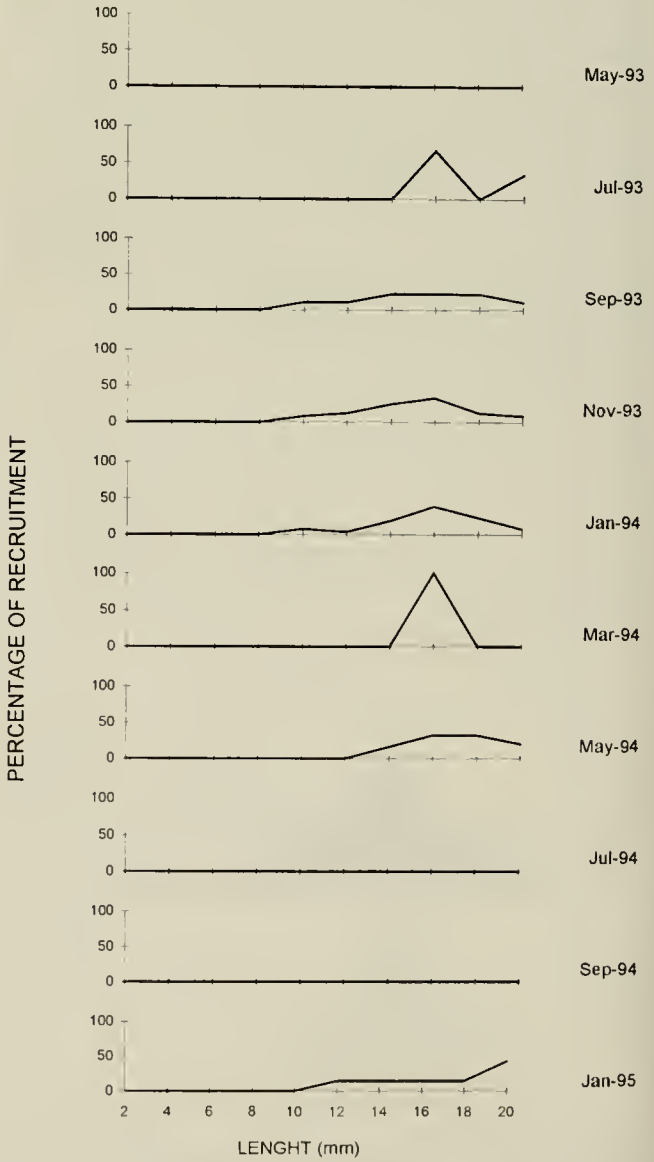

Figure 7. Bimontly percentage of recruitment for purple snail.

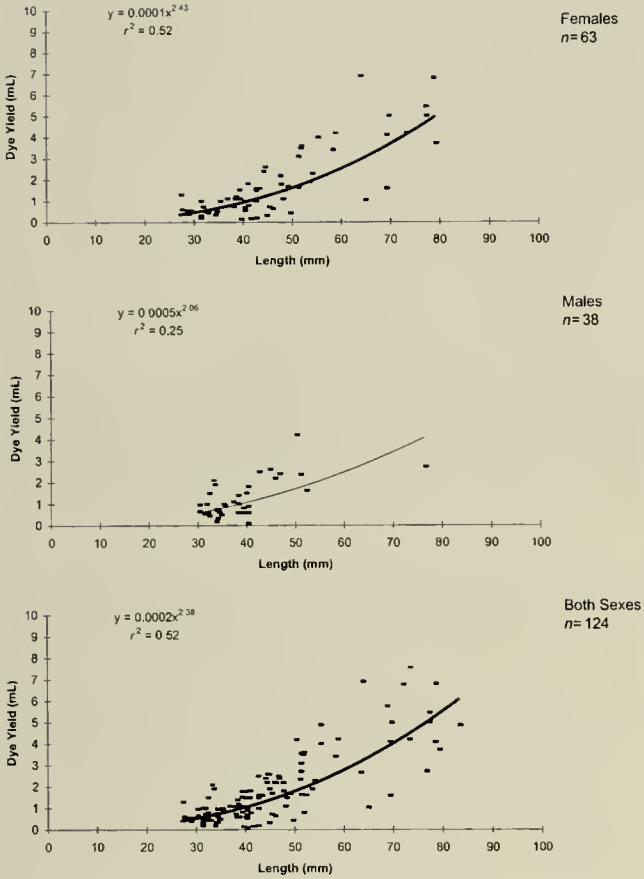

Figure 8. Length-dye yield relation of female, male, and unsexed (both) snails *Plicopurpura pansa.*

TABLE 4.

Estimated power regression values ($Y = aS^b$) between snail size (S, mm) and dye yield (Y, mL) for purple snail *Plicopurpura pansa.*

Attribute	Females	Males	Total[a]
a	.0001	.0005	.0002
b	2.43	2.06	2.38
r^2	.52	.25	.52
n	63	38	124

[a] Total includes female, male, and unsexed snails.

DISCUSSION

To compare the purple snail with other mollusks, in Table 5 estimates of growth parameters ($L\infty$ and K of the VBGM) for several species of gastropods were compiled. These parameters were compared among gastropods following the methods of Pauly and Binohlan (1966). Data of $L\infty$ and K for those species and those of the purple snail were plotted in auximetric grids shown in Figure 9; in addition, $L\infty$ and longevity (as 3/K) is also displayed in Figure 10.

These comparisons show that the purple snail displays a quite different strategy from other species because it has a slow growth rate and is small compared to other species. Although it is small, it lives longer than the others, which could be a result of its predatory activity and mobility in the very limiting environment of the intertidal zone, where animals with a small size may have a better chance of survival struggling against the impact of waves.

On comparing our own results with other growth estimates by graphic methods (Battacharya's), the results are different. K values obtained seem to be low (.069 and .088 for females, after Turok et al. 1988, Alvarez 1989). These values suggest great longevity and are in contrast with estimates obtained by tagging methods by the same authors (2 mm/mo). However, $L\infty$ estimates are similar to our estimates.

TABLE 5.

Estimates of growth parameters (L, K, and t_0) for the purple snail and several other species of gastropod mollusks.

Common Name	Species	$L\infty$ (mm)	K	t_0	Source
Panocha	*Astraea undosa*	103	0.12	—	Cupul–Magaña and Torres–Moye (1996).
Loco	*Concholepas concholepas*	150	0.32	—	Stolz and Pérez (1992).
Blue abalone	*Haliotis fulgens*	189	0.34	—	Guzmán del Próo et al. (1976).
		170	0.36	–0.05	Guzmán del Próo et al. (1980).
		183	0.38	—	Shepherd et al. (1991).
		175	0.24	–0.43	Turrubiates and Castro–Ortiz (1992).
		177	0.28	–0.24	Turrubiates and Castro–Ortiz (1992).
		190.2	0.37	—	Shepherd and Turrubiates (1997).
		182.6	0.36	–0.36	Shepherd and Turrubiates (1997).
		187.1	0.35	—	Shepherd and Turrubiates (1997).
		179.6	0.29	–0.78	Shepherd and Turrubiates (1997).
		126	1.38	–0.07	Shepherd and Turrubiates (1997).
Queen Conch	*Strombus gigas*	260	0.51	0	Randall (1964).
		201	0.59	0	Berg (1976).
		296	0.42	–0.05	Berg and Olsen (1989; after Alcolado, 1976).
		318	0.38	–0.08	Hesse (1976).
Purple	*P. pansa*				
	Males	102	0.21	–0.04	This paper
	Females	110	0.27	–0.04	This paper

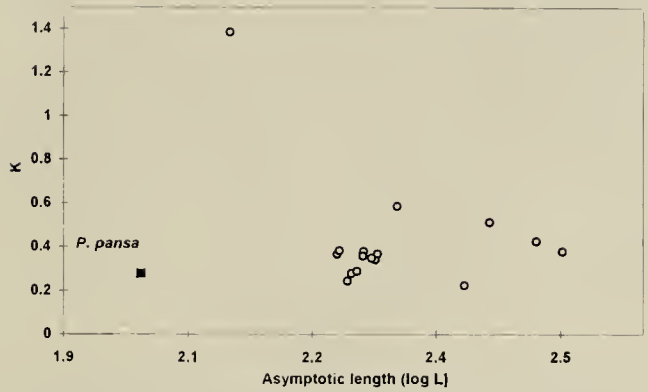

Figure 9. Auximetric grid showing growth performance expressed as the parameters K and L∞ of the VBGM, of the purple snail (*P. pansa*) as compared to the same parameters for the blue abalone (*Haliotis fulgens*), the loco (*Concholepas concholepas*), the queen conch (*Strombus gigas*), and the wavy turban (*Astraea undosa*).

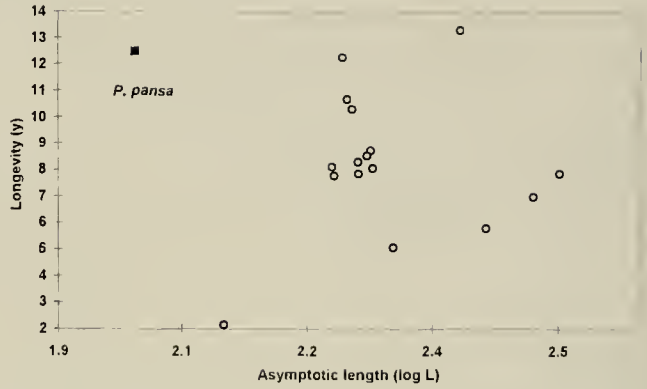

Figure 10. Same as Figure 9, where L∞ and longevity (determined as 3/K) of the purple snail (*P. pansa*), are compared to the same parameters of the blue abalone (*Haliotis fulgens*), the loco (*Concholepas concholepas*), the queen conch (*Strombus gigas*), and the wavy turban (*Astraea undosa*).

Though several methods are available and useful (Devillers et al. 1998), we used the VBGM to compare our estimates with previous works on the purple snail and with other gastropods. Moreover, in this generally applicable model, parameters have biological significance because they are based upon metabolism, not only on mathematical aspects (Bustos et al. 1986).

The mortality of the purple snail is evaluated here for the first time. After mortality estimates were found by several methods, ranging from 0.21 to ≈ 0.5, it led us to believe the values obtained by the catch-curve and the Jones and van Zalinge methods are probably overestimates because of reasons intrinsic to each method. These differences could be caused by changes in spatial distribution of snail population, which are reflected as changes in length–frequency distributions and considered as mortality by the catch-curve and by the Jones and van Zalinge methods.

Sexual proportion and density could be affected by changes in spatial distribution of the purple snail. In the laboratory and in the field, we observed some changes in relation with reproductive success—aggregations of males around a female—however, in this work no gradient by size was observed and the sampling method was not specific for spatial distribution determination.

The information in this work on the population dynamics and dye yield of the purple snail can be considered as baseline data and a first step to answer the question whether a fishery for purple dye extraction can be established or not. Moreover, this allows us to look for specific research required in distinguishing critical points in this activity and about the snail population. Questions as to the effect of repetitive dye extraction over dye and survival rate, or physiological role of the dye, should be studied. With this information, recent approaches in fishery biology and resource management, i.e., simulation models, could be used to assess the best management strategies of this ancient resource from a modern viewpoint.

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TECHNIQUES FOR ASSESSING REPAIRED SHELL DAMAGE IN DOG COCKLES *GLYCYMERIS GLYCYMERIS* L.

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ABSTRACT Three techniques for assessing repaired shell damage in dog cockles *Glycymeris glycymeris* (visual assessment, x-rays, and shell sectioning) were examined for objectivity and repeatability. Visual assessment of the number of scars was found to be inaccurate and highly subjective. Image analysis of x-rays suffered from inconsistencies in film development and image quality. However, this technique provided an estimate of the area of the shell affected by scarring, rather than simply a record of the number and/or severity of scars. Examination of shell cross-sections provided the most objective and repeatable technique, although the methodology had a number of disadvantages as it was time consuming and scar severity could be underestimated depending on the position of the line of section through the shell. This technique also offers the opportunity to date the formation of the scars using internal annual growth lines.

KEY WORDS: Shell damage, *Glycymeris glycymeris*, x-rays, shell sections, visual assessment

INTRODUCTION

Repaired shell damage, or scarring, in molluscs has been used to provide information about a range of biotic and abiotic disturbances. In this paper scars are defined as indentations in the shell surface where the shell margin has apparently been chipped and subsequently repaired. In gastropods, shell damage has often been used to infer predator activity both in modern populations and those from the palaeontological record (Vermeij et al. 1981, West et al. 1991, Cadée et al. 1997). Several studies have investigated the role of fishing disturbance by towed demersal gears (trawls or dredges) in causing non-lethal shell damage in both gastropods and bivalves (Gaspar et al. 1994, Witbaard and Klein 1994, Mensink et al. 2000, Ramsay et al. 2000). These studies have suggested that it may be possible to use repaired shell damage as an indicator of fishing intensity.

If repaired shell damage is to be used to infer levels of historical disturbance (either natural or anthropogenic), a reliable method for quantifying this damage is required. Previous investigators have largely relied on a visual assessment of shell damage and this technique has been successfully used for gastropods (Preston et al. 1993, Cadée et al. 1997, Mensink et al. 2000). For the bivalve *Glycymeris glycymeris* (L.) however, visual assessment presented difficulties, as minor scars could not be readily distinguished from marks of annual origin in an objective manner (Ramsay et al. 2000).

G. glycymeris is a largely infaunal bivalve and is commonly found in gravelly sediments, although animals are also found in muddy and sandy sediments (Tebble 1966). The depth to which these animals bury appears to vary according to the substratum type, with the deepest depths of several centimeters being reached in gravel (Ansell and Trueman 1967). Shells of *G. glycymeris* often exhibit signs of repaired damage and Ramsay et al. (2000) found a correlation between the occurrence of scarring and fishing effort around the Isle of Man, Irish Sea, whilst Steingrímsson (1989) suggested that scars may be caused by unsuccessful predator attacks or storm damage. In this paper we have compared the accuracy and repeatability of three methods for assessing shell scars in *G. glycymeris*: (1) visual assessment, (2) image analysis of x-rays, and (3) examination of shell cross-sections.

MATERIALS AND METHODS

A sample of 40 live *G. glycymeris* was collected from a site off the East Coast of the Isle of Man (water depth of 50 m, sediment of mainly gravel and coarse sand) in October 1997. Repaired shell damage (scarring) was assessed in dry, clean *G. glycymeris* shells (shell height [maximum measurement from the dorsal to the ventral edge] 4–5 cm) from which the periostracum had been removed by gentle brushing.

Visual Assessment

A six-point damage scale (Table 1) was used by two assessors (with prior experience of working with *G. glycymeris*) and three small groups of up to five students to categorize damage in the shells. Agreement between recorders was analysed using a generalization of Cohen's kappa statistic (Fleiss 1971, Banerjee et al 1999).

Image Analysis of X-Rays

Shells were x-rayed (height of the x-ray source above the shells 75 cm, power and exposure of 60 kV for 0.04 sec), 20 shells each time on a photographic film measuring 30 × 24 cm. One shell was

TABLE 1.

The scale used for the visual assessment of shell scars.

Score	Description
0	No scars
1	Very mild damage, e.g. 1 small scar
2	Mild damage, 2 or more small scars
3	Moderate damage, 1 larger scar, possibly also small scars
4	Moderate/severe damage, several large scars
5	Severe damage, lots of large scars or large chunks missing

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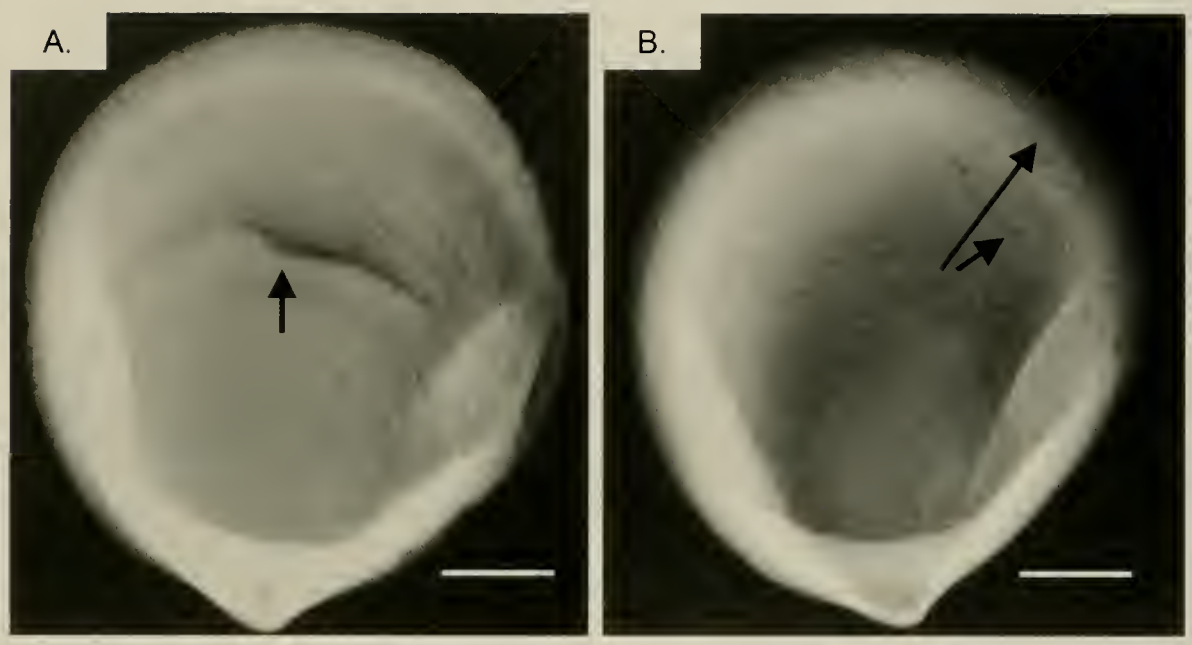

Figure 1. Shell x-rays. (A) Area of repaired damage indicated by arrow. (B) Poor resolution created difficulties in distinguishing areas of repaired damage. Scale bar = 1 cm.

included on both x-ray sheets to allow calibration in the case of inconsistencies in film development. The damaged areas of the shell (scars) (as seen in visual inspections) appeared as darker areas on the x-ray (Fig 1a). These x-ray images were captured as digital images and each image was examined using image analysis software (*Sigma Scan*, Jandel Scientific). Possible scars were identified by eye and greyscale measurements (a measurement of darkness/lightness with a scale of 0 (nearly black) to 255 (nearly white) [Jandel Scientific Software, 1995]) were taken along a line of eight points within the scar and eight points either side of the scar (Fig 2). Wherever the difference between the average greyscale values (within the scar and outside the sear) exceeded 15, the area that was as dark or darker than the within-scar average was measured. The surface area of the shell was also determined using image analysis and the proportion of the shell that was damaged calculated.

Figure 2. Procedure for determining shell scars from x-rays. The area was considered to be a scar if the difference between the average greyscale values within the scar (central line of dots) and outside the scar (outer two lines of dots) exceeded 15. Scale bar = 1 cm.

Examination of Cross-Sections

Shells were embedded in resin and sectioned using a diamond saw along a line from the umbo to the centre of the ventral edge. The section containing the posterior portion of the shell was

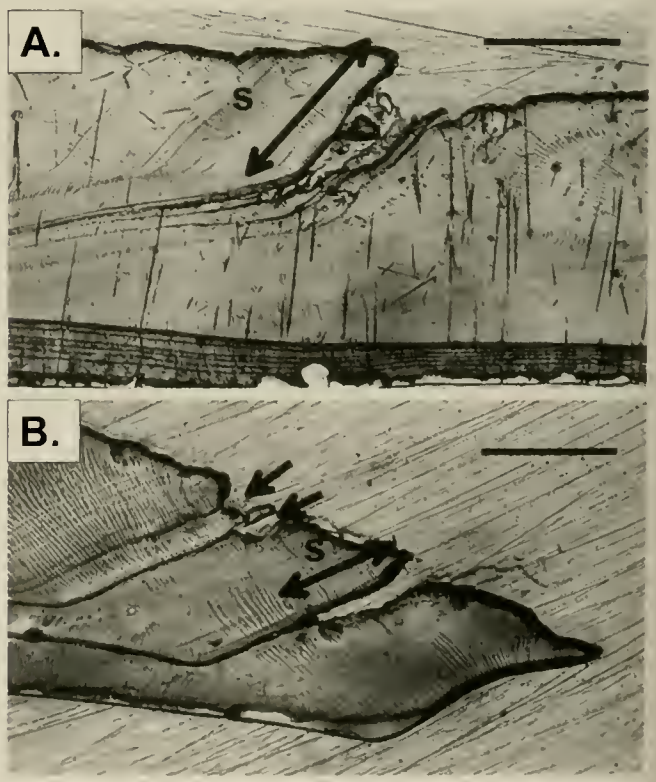

Figure 3. Acetate peels of shell scars viewed in cross-section. Line(s) is the measurement of the scar depths. A was estimated to be 19-years-old with a scar at the age of 9-years-old; and B is 19-years-old, scars at 19, 18, and 17 years (17- and 18-y scars indicated by arrows). Scale bar = 0.5 mm.

ground smooth, polished, and etched for 3 min using 0.1 M hydrochloric acid. Acetate peel replicas were prepared of the cross-sectioned surface (Kennish 1980) to allow microscopic examination of shell damage and internal growth lines (Fig. 3). Repaired shell damage was visible in these acetate peels as an indentation in the normal growth line of the outer shell surface (Fig 3).

Since the shell was only sectioned along the line of maximum growth, estimates of shell damage through this single cross-section might lead to scars being missed if they occurred elsewhere around the shell margin. However, it seems likely that severe damage to one part of the shell edge would result in a growth disturbance across the entire shell margin. To test this hypothesis, a sample of

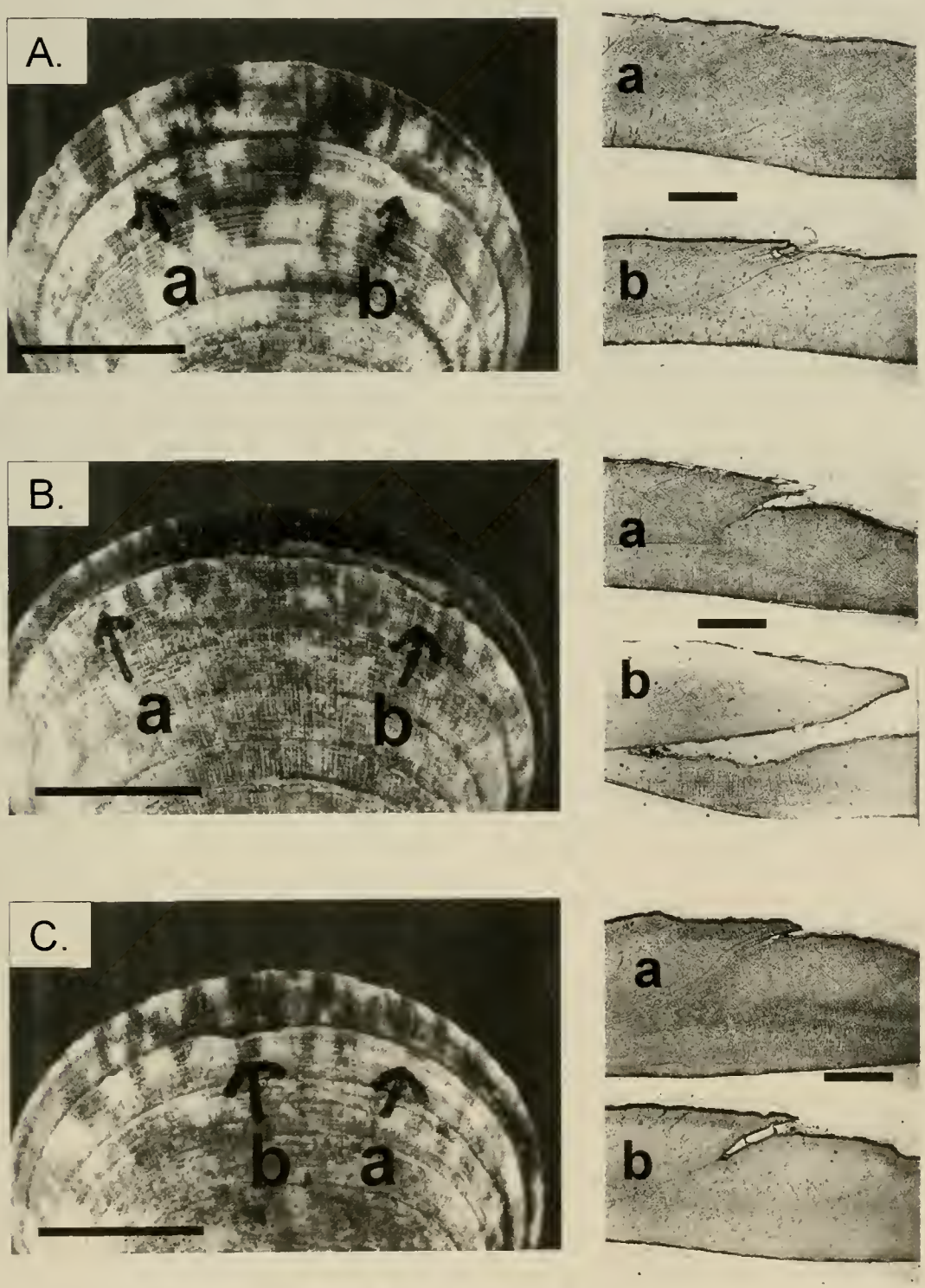

Figure 4. Photographs of shells with repaired damage showing the two lines of sectioning through the most severely damaged and least damaged areas. Scale bar = 1 cm. The corresponding cross-sections are shown to the right of the shell photographs. Scale bar = 0.5 mm. Each figure corresponds to the following shell number on Table 2: A, Shell 1; B, Shell 3; and C, Shell 5.

10 live animals (23–36 mm shell height) that had visible chips around the shell edge were selected from a sample collected by dredging. These shells were placed in a laboratory upwelling system (Spencer 1988) for 9 mo to allow the animals to grow and repair the shell damage. Shells were cleaned and dried, photographed (to record the appearance of the shell surface), embedded in resin, and sectioned along two directions from the umbo to the shell margin. The first section was placed through the centre of the most severely damaged area, whilst the second section was placed through an apparently undamaged region (Fig 4). Acetate peel replicas of the two polished shell sections were prepared and the dimensions of the damage were compared.

RESULTS

Visual Assessment

Researchers found it extremely difficult to quantify scars through a visual assessment, as it was difficult to distinguish between smaller scars and annual growth lines on the shell surface and this led to the development of the damage scale. However, visual assessment using the damage scale in the 40 shells was still highly variable with only one shell receiving the same score in all five assessments and two shells being given the same score by four out of the five assessments. The kappa statistic returned a value of 0.07 (perfect agreement ≡ 1, a random set of numbers ≡ 0, and agreement between 3 assessments for every shell ≡ 0.16). When analysis of shell damage was restricted to the assessments of the two researchers who had substantial prior experience of working with *G. glycymeris* shells, the kappa statistic was still low (0.25; 52% of shells received the same score from both researchers).

Image Analysis of X-Rays

The x-ray images generally had poor resolution or "blurring" of the shell margins (Fig. 1, a and b). For this reason, the number of scars identified by the x-ray method was generally low (mean 1.2 ± 0.2 scars per shell). The black-and-white contrast appeared to vary between the two sheets, probably as a result of inconsistencies in x-ray development. However, the inclusion of a "standard" shell on each sheet helped to minimise, but not eliminate, these problems.

Examination of Cross-Sections

Shell damage was evident in many (95%) of the 40 acetate peels of shell cross-sections as a break in the continuity of the shell edge (Fig. 3). The depth of these breaks, or scars, could easily be measured and these measurements proved to be repeatable between recorders with a small degree of error (the average error between two recorders who measured 30 scars was 16 μm [scar sizes ranged from 25 –1,150 μm]). The average number of scars per shell was 2.4 ± 0.6 for scar sizes greater than 250 μm and 3.2 ± 0.8 for scars between 125 and 250 μm. Thus more scars were identified using this methodology than using the x-ray technique.

The experimental shells from the upwelling system had repaired the shell margin chips by the end of the 9-mo experimental period. The repaired shell damage was apparent in cross-sections taken along two directions, including the one through the region that appeared undamaged from a visual inspection. However, the scars from the visually undamaged area were smaller than those of the damaged section (Table 2 Fig 4).

TABLE 2.

Depth of scars from shells damaged by a dredge. Sections were cut through the area of worst damage and an apparently undamaged area (from visual inspection). See Figure 4 for photographs and acetate peels of shells 1, 3, and 5.

	Scar Depths (μm)	
Shell nn.	Worst damage	Least damage
1	175	150
2	250	225
3	2,350	675
4	200	100
5	725	300
6	175	150
7	550	250
8	150	150
9	100	50
10	650	75

DISCUSSION

The results demonstrated that analysis of acetate peels of shell cross-sections was the most reliable and repeatable method for quantifying repaired damage in the shells of *G. glycymeris*. Shell damage gives rise to a recordable growth anomaly around the entire shell margin, although the anomaly was most pronounced in the area where damage appeared most severe from an external visual inspection. The most accurate method for assessing the frequency of shell damage might be to produce multiple cross-sections through each shell (although this would be highly time consuming [about a 35-min preparation per section]). This technique also offers the opportunity to date the formation of the scars using the internal annual growth lines (Witbaard and Klein 1994, Ramsay et al 2000).

X-rays also proved to be a potentially useful method of assessing the frequency of shell scars, although this method tended to pick up fewer scars in comparison with the analysis of cross-sections. However, the technique has the advantage of analysing the entire shell rather than a single cross-section and estimates shell damage as a percentage of the total shell surface area, unlike the acetate peel technique, which can only quantify the number of scars. The method is also non-destructive, which may be useful when analysing valuable palaeontological or archaeological samples. X-rays of bivalve shells have also been used successfully to assess the extent of infestation by shell-boring parasites (Ambariyanto and Seed 1991).

Visual assessment of shell scars in *G. glycymeris* tended to be unreliable with poor repeatability compared with the other two techniques. However, it is possible that visual inspection may be useful if an attempt were made to differentiate between causes of scarring (e.g. predator attacks, fishing disturbance, and/or storm damage) from the appearance of scars, although this has proved difficult to date (unpublished data).

It appears that microscopic examination of shell cross-sections is the most reliable method for quantifying repaired shell damage in *G. glycymeris* and this could possibly be used in conjunction with a visual assessment to provide additional information about the nature of the scars. The combination of x-rays and image analysis may also be a technique worthy of further development and could also be used in conjunction with the analysis of shell cross-sections to allow an initial count of the number of scars followed by a measurement of the area covered by the more severe scars.

ACKNOWLEDGMENTS

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POPULATION BIOLOGY OF GAPER (HORSE) CLAMS, *TRESUS CAPAX* AND *T. NUTTALLII*, IN SOUTHERN BRITISH COLUMBIA, CANADA

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ABSTRACT Growth and mortality rates, and densities of gaper (horse) clams, *Tresus capax* and *T. nuttallii*, were estimated from several areas in southern British Columbia (B.C.). Growth rates of *T. capax* from low intertidal and subtidal zones were greater than those from mid intertidal zones reported in other studies in B.C. Growth rates for *T. nuttallii* varied between areas. Mean natural mortality rates for adult *T. capax* were .15 to .20 from Seal Island, and for adult *T. nuttallii* were .44 from Ritchie Bay and .20 from Klaskino Inlet. Densities and biomass of *T. capax* were higher in the low intertidal zone than the subtidal zone at the Seal Island study area and for *T. nuttallii* in the subtidal at Ritchie Bay. The relative abundance of *T. capax* and *T. nuttallii* in the subtidal varied considerably between locations.

KEY WORDS: horse clam, fat gaper, *Tresus capax*, Pacific gaper, *T. nuttallii*, growth, mortality, density

INTRODUCTION

The two horse clam species, the fat gaper, *Tresus capax* (A. A. Gould 1850) and Pacific gaper, *T. nuttallii* (Conrad 1837) (Bivalvia: Mactridae), are found from Alaska to California and are common in mud, sand, and gravel substrates along British Columbia (B.C.) coastal waters (Quayle 1960, Bernard 1983, Coan et al. 2000). *Tresus capax* is found from mid-intertidal beach levels to subtidal depths of at least 20 m (Bourne and Smith 1972b, Haderlie and Abbott 1980), whereas *T. nuttallii* is found from the low intertidal to subtidal depths of 50 m (Haderlie and Abbott 1980, Campbell et al. 1990, Coan et al. 2000). Both species are commercially harvested subtidally (at depths >3 m) in B.C.; this modest fishery (started in 1979 with landings of 37t and ranged from 355t in 1987 to 3t in 1995) has been limited due to lack of markets for the processed product and lack of stock assessment (Harbo and Hobbs 1997, Lauzier et al. 1998). Although industry has requested expansion of this fishery, management has resisted increase in exploitation until further information on abundance and biology of these two species was obtained (Lauzier et al. 1998). Some information is available on the biology and abundance of *T. capax* and *T. nuttallii* in B.C. (Quayle 1960, Quayle and Bourne 1972, Bourne and Smith 1972a, Bourne and Smith 1972b, Bourne and Harbo 1987, Campbell et al. 1990, Bourne and Cadwell 1992, Rice et al. 1993, Bourne et al. 1994). Data on horse clam biology from other areas come mainly from subtidal populations of *T. nuttallii* (Harrington and Griffin 1897, MacGinitie 1935, Swan and Finucane 1952, Fitch 1953, Addicott 1963, Armstrong 1965, Pohlo 1964, Pearce 1965, Smith and Davis 1965, Stout 1967, Stout 1970, DesVoigne et al. 1970, Laurent 1971, Clark 1973, Clark et al. 1975, Kvitek et al. 1988) and mostly from intertidal and a few subtidal populations of *T. capax* (Pearce 1965, Pearce 1966, Reid 1969, Machell and DeMartini 1971, Stout 1967, Stout 1970, Armstrong and Armstrong 1974, Wendell et al. 1976, Gaumer 1977, Goodwin and Shaul 1978, Breed-Willeke and Hancock 1980, Robinson and Breese 1982, Kvitek and Oliver 1992). A third species, the strange gaper, *T. allomyax* Coan & Scott, which Coan et al. (2000) indicated was incorrectly named *T. pajaroanus* (Conrad, 1875) by Dinnel and DeMartini (1974), has a limited distribution from Oregon to California.

The purpose of this paper is to present estimates on density, growth, and mortality of inter- and sub-tidal *T. capax* and subtidal *T. nuttallii* populations, which will be useful in fishery management of these species in B.C.

MATERIALS AND METHODS

Horse clam densities were estimated from a study plot in Ritchie Bay, northwestern Meares Island, near Tofino (Lat. 49°13.43′N Long. 125°54.99′W) during June 3–9, 1993, and in the northwestern side of Seal Island (Islets), near Comox (Lat. 49°37.835′N Long. 124°51.892′W) (Fig. 1) during June 3–7, 1993, between the low intertidal depths of −1 m to subtidal depths of about 10 m subtidal for Ritchie Bay and 11 m for Seal Island. Study plots were characterized by sand and broken shell substrates at all depths. Eelgrass (*Zostera marina* L.) abundance, in shallow water, was dense at Ritchie Bay and low at Seal Island. Both study plots, 0.5 ha in area (50 m × 100 m), were delineated with lead lines and subdivided by 10 (50 m) transect lines 10 m apart running from shallow to deep. The survey of horse clam density involved counting horse clam siphons showing at or above the substrate in 5 m^2 (5 × 1 m) quadrats along both sides of the inner subdivision transect lines and on the inner sides of the study plot boundaries. Each transect was assumed to be a sample with quadrats as secondary sample units. Depths recorded by divers at each quadrat were corrected for a standard tidal height at datum (mean low lower water) at the time (±5 min) each quadrat was sampled. Density data for the west boundary of the Seal Island study plot were missing for some unknown reason.

Samples for size and age frequency distributions and growth estimates were obtained by randomly collecting horse clams within two depth zones (shallow 2 to 3 m and deep 4 to 10 m subtidal) in the Ritchie Bay study plot and (low intertidal −1 to 1

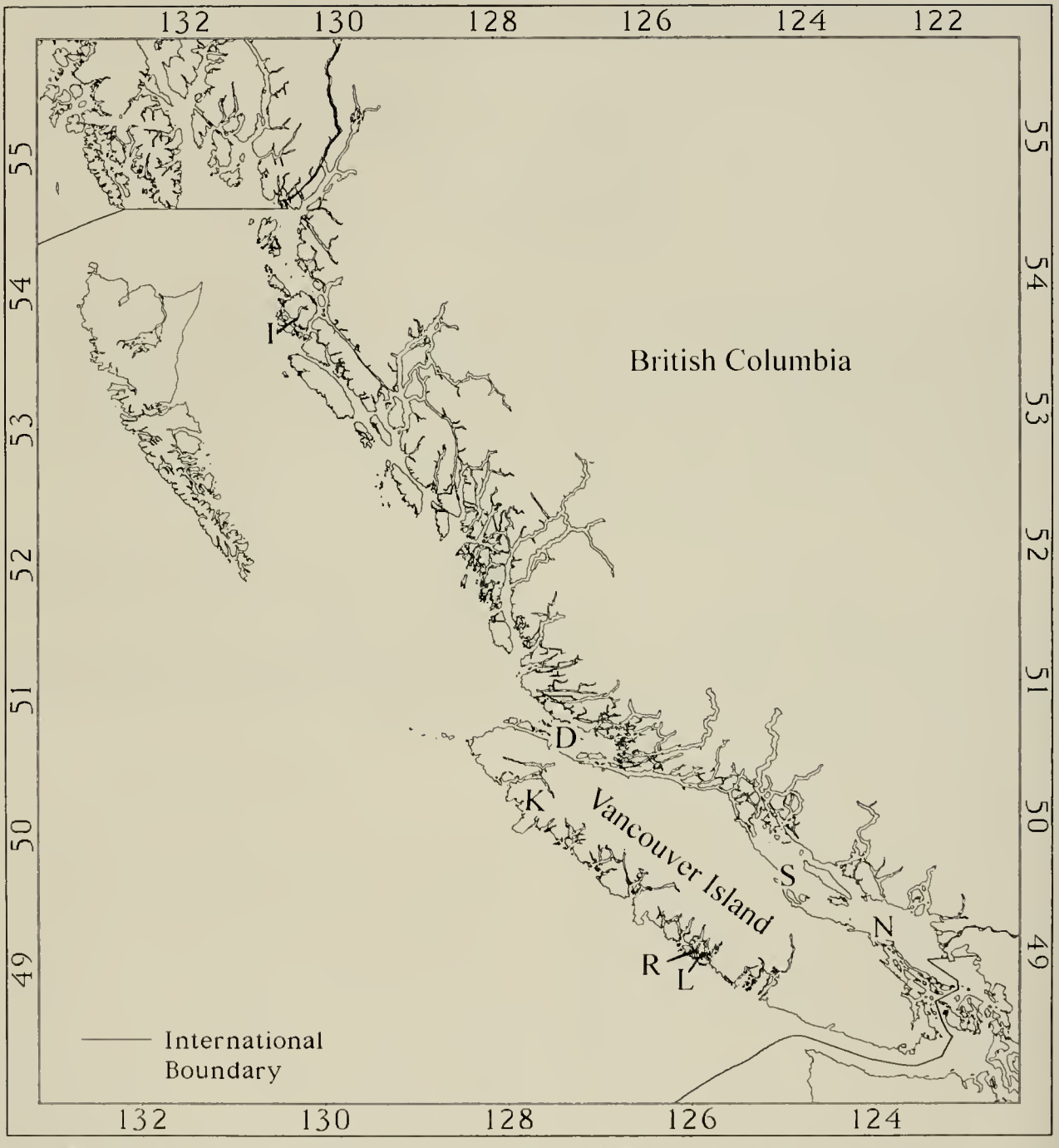

Figure 1. Map of British Columbia indicating general sample locations. D = Doyle Island, I = Kitkatla Inlet, K = Klaskino Inlet, L = Lemmens Inlet, N = Newcastle Island, R = Ritchie Bay, S = Seal Island or Seal Island.

m and subtidal 2 to 11 m) in the Seal Island study plot. Horse clams were hand collected with a diver operated "stinger" (1-m long stainless steel pipe, attached to a high pressure water hose, that provides a water jet to allow horse clam removal from the substrate) (Goodwin 1973). A commercial sample of horse clams was obtained from Klaskino Inlet (L. Lat. 50°17.9′N Long. 127°48.8′W) on June 23, 1993. An additional sample from as wide a size range as possible of *T. capax*, to determine growth, was obtained from Lemmens Inlet (Lat. 49°12.2′N Long. 125°52.3′W) May 25 and August 10, 1989.

Sampling methods, for counting density or collecting individual animals, in this study relied on visual detection of horse clam siphons in the substrate under water by experienced commercial dive fishers, which generally only included horse clams of commercial size (>100 mm shell length, SL). Consequently density estimates should not be considered as absolute but rather minimum estimates of abundance. Small horse clams (especially <50 mm SL) were usually difficult to see and collect by divers and a few horse clams may not have shown their siphons clearly above the substrate surface. Additional samples of *T. capax* (11–50 mm

SL) were obtained, for dissection, by using a venturi dredge in the Seal Island study plot.

For each collected horse clam, SL was measured as the straight line distance between the anterior and posterior margin of the shell to the nearest 1 mm with vernier calipers, total wet weight and shell weight were recorded to the nearest 0.1 g. In addition, whenever possible subsamples, from as wide a SL range as possible, were obtained from horse clams collected in each area and wet weights of the drained total body and shell, shell only, whole soft body and siphon (neck) only (cut at base of siphon) were recorded to the nearest 0.1 g within 24 h of collection. For Klaskino Inlet, only the total wet weight (Wt), shell length, and weight were recorded for each individual animal; total drained weight (Wd) was estimated as Wd = Wt C, where C is the mean free-water weight loss conversion ratio (Wd/Wt = 0.74) calculated from horse clams from the other areas in this paper.

Age of horse clams was determined by counting the number of annuli on the shell, and growth was determined by measuring shell length at each annulus after Weymouth et al. (1925) and discussed by Bourne and Smith (1972b). Horse clams had pronounced annuli up to about 20 years of age; a few clams older than 20 years had annuli spaced close together and accuracy in age determination of the clams was estimated at about ±2 y. Horse clams with broken shells were discarded.

Mean density, d (number / m^2), was calculated as

$$d = \frac{\sum_i c_i}{\sum_i a_i}$$

Standard error of the mean density, se(d), was calculated as

$$se(d) = \sqrt{\frac{\sum_i (c_i - da_i)^2}{n(n-1)a^2}}$$

where for each i^{th} transect, c_i is the number of horse clams observed in a transect, a_i is the area of transect surveyed in square metres, a is the mean transect area for all transects and n is the number of transects sampled. This method was also used to calculate mean and standard error of density from a depth interval by subsampling each transect in the particular depth range (i.e., (1) ≤ 0.0 m, (2) >0.0–2.0 m, (3) >2.0–3.0 m, (4) >2.0–4.0 m, (5) >4.0–6.00 m, (6) >6.0–8. 0 m, (7) >8.0–10.0 m, and (8) >10.0–12.0 m).

Total mortality rate (Z) was estimated in the usual way (Ricker 1975) by calculating the slope of the regression relationship between the natural log of the frequency and age of horse clams >10 y, which would include mature (Bourne and Smith 1972b, Campbell et al. 1990) horse clams fully recruited to the fishery and mainly the descending right limb of the age frequency curve. We assumed that the mortality rate reflected the natural mortality rate because most of the age frequencies of horse clams sampled from areas that had little or no commercial fishing history were used. Ritchie Bay was designated as a research study area since the early 1980s and has had no commercial dive fishing activity for horse clams. Although Seal Island is in an area (bed 4801) where horse clam landings have been recorded from the subtidal (dive fishing for horse clams in waters shallower than 3-m depths is prohibited to protect eelgrass habitat) (Harbo and Hobbs 1997), fishing for horse clams in the low intertidal areas of Seal Island probably has not occurred. An insignificant amount of harvested horse clam landings have been reported from Klaskino Inlet (statistical management area 27) (Harbo and Hobbs 1997). Lemmens Inlet (statistical management area 24) has had considerable amounts of horse clams harvested (Harbo and Hobbs 1997) and were not used to estimate mortality rates because of low sample sizes.

Average von Bertalanffy growth curves were fitted to data points of size at age using the equation:

$$L_t = L_\infty (1 - e^{-k(t - to)})$$

where t is age in years, L_t is the shell length at t, L_∞ is the theoretical maximum size, k is a constant, determining rate of change in length increments, and t_0 is the hypothetical age at which

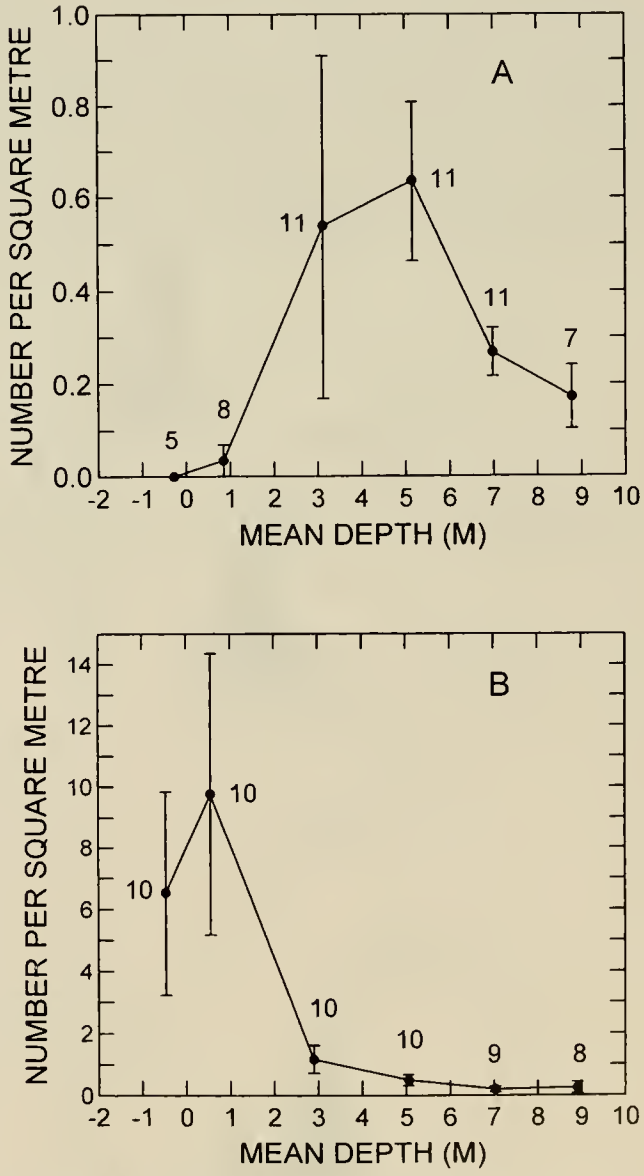

Figure 2. Mean density of horse clams by depth at the study areas in (A) Ritchie Bay (99.2 % were *T. nuttallii* at all depths) and (B) Seal Island (*T. capax* were represented 100% in <2 m depths and 99.5% in >2 m depths). Vertical lines are ± 2 SE; numbers beside dots are number of transects represented at each depth range.

the organism would be at zero length. The parameters L_∞, k, and t_0 were estimated using a nonlinear least squares method (SYSTAT 1996).

Allometric relationships between total, body, neck and shell weights (Y), and shell length (X) were determined using the power equation of the linear form $\log_e Y = \log_e a + b \log_e X$, where a and b are constants calculated using the least squares method. Comparison between sampled areas for each relationship was accomplished testing for homogeneity between slopes and subsequently comparing intercepts of lines by adjusting the Y variables and testing for differences by analysis of covariance (ANCOVA) using shell lengths or age as covariates (Snedecor and Cochran 1967) with SYSTAT.

Mean weight of the size frequency sample was calculated by $\Sigma(N_L W_L)/\Sigma N$, where N_L is the number of animals per SL interval, ΣN is the total number of animals in the size frequency sample, and W_L is the predicted mean weight for a particular SL estimated from power equations. For Klaskino Inlet, the total wet weight of each individual horse clam was measured and the drained total wet weight was calculated by multiplying the total wet weight by .74 (the mean free-water weight loss conversion ratio). The estimated mean biomass per m^2 was calculated as the product of the mean weight and the mean density of horse clams in the study area.

RESULTS

Density

At Ritchie Bay, density was higher in the 3–6 m range than at other depths sampled; few horse clams were found in the intertidal <1 m depth, especially in the dense eelgrass (Fig. 2A); most horse clams sampled (99.22%, n = 511) were *T nuttallii*. At Seal Island, densities were highest at the low intertidal and <1 m depth and were low at >2 m depths (Fig. 2B) (no horse clams were found at 10.5 m, n = 4); *T. capax* represented 100% in samples (n = 525) from the shallow waters and 99.50% (n = 602) from >2 m depths. Overall mean density of horse clams was lower at Ritchie Bay, 0.32 per m^2 (±0.03 SE, n = 11), than at Seal Island, 4.54 per m^2 (± 0.85 SE, n = 10). Distribution of density changed with depth at both study areas (Fig. 2).

Size and Age Distributions

Most horse clams sampled were between 100 and 200 mm SL (Fig. 3). Average size and age were higher for *T. nuttallii* than for *T. capax* (Figs. 3 and 4). The largest (230 mm SL) and oldest (24 y) *T. nuttallii* were from Klaskino Inlet (Fig. 3B and Fig. 4B). The largest (187 mm SL) and oldest (21 y) *T. capax* were from Seal

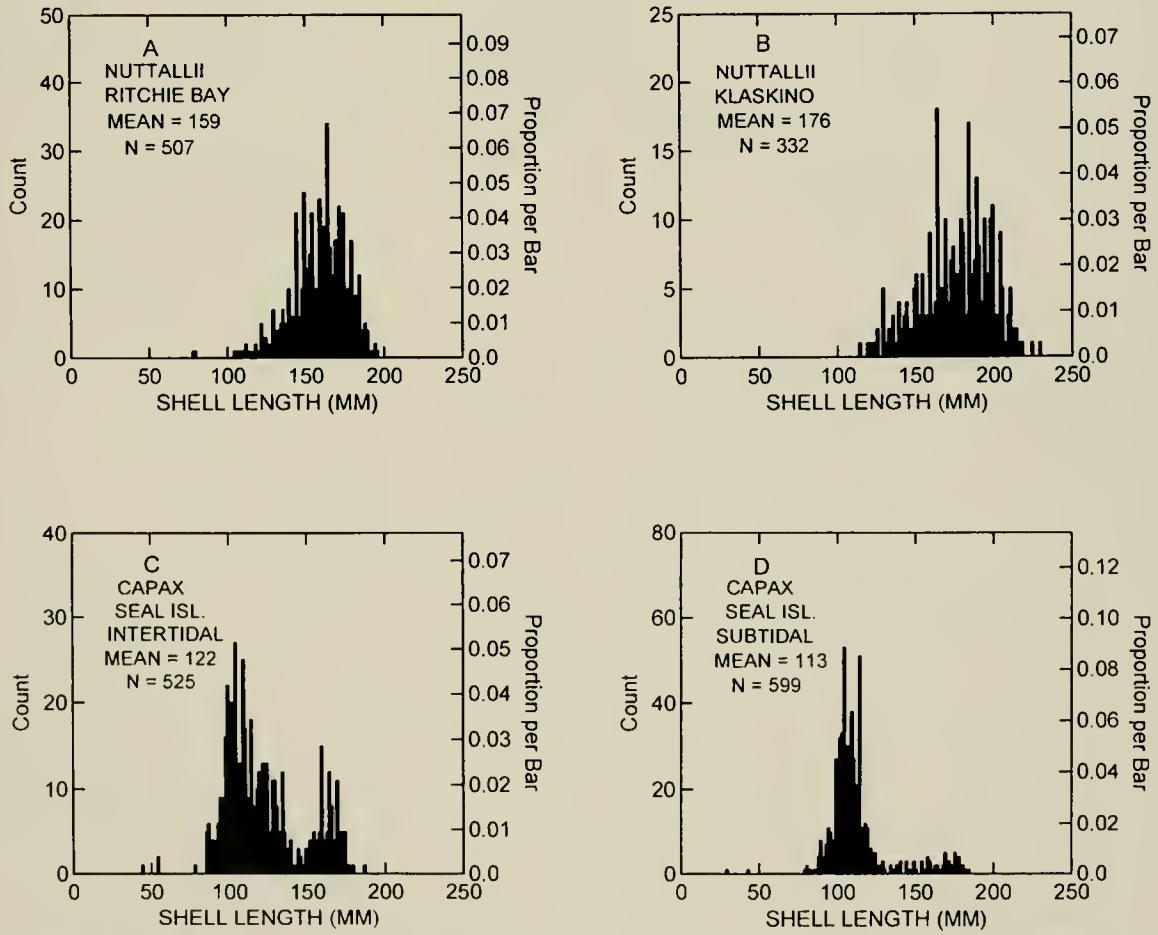

Figure 3. Size frequency distributions for (A) *T. nuttallii* at Ritchie Bay, (B) *T. nuttallii* at Yellow Bank, (C) *T. capax* Seal Island from low intertidal zone to 1m depth, (D) *T. capax* Seal Island, (E) *T. nuttallii* at Klaskino Inlet location 1, and (F) *T. nuttollii* at Klaskino Inlet, locations 2 and 3 combined. All samples are from subtidal (>2 m depth) locations except the intertidal sample at Seal Island (C).

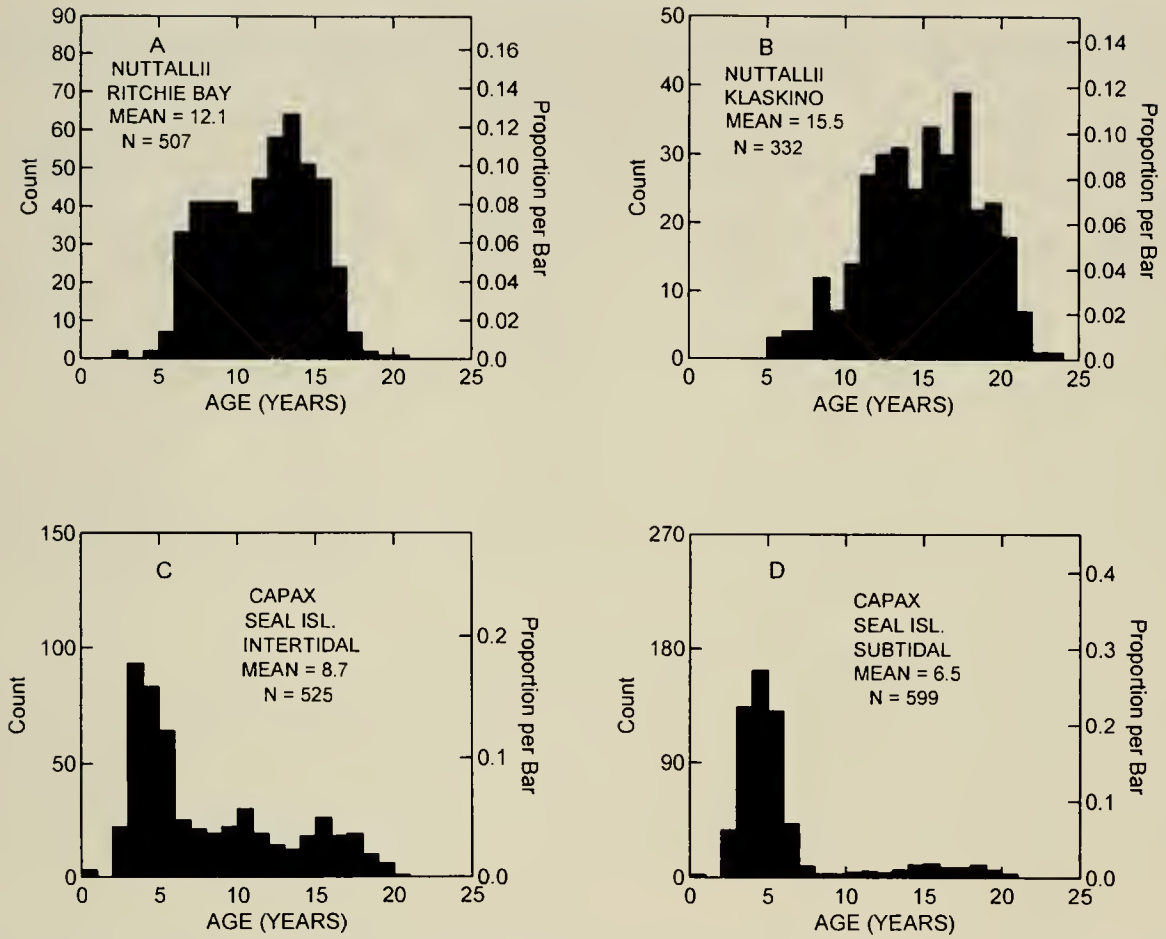

Figure 4. Age frequency distributions for (A) *T. nuttallii* at Ritchie Bay, (B) *T. nuttallii* at Yellow Bank, (C) *T. capax* Seal Island from low intertidal zone to 1m depth, (D) *T. capax* Seal Island, (E) *T. nuttallii* at Klaskino Inlet location 1, and (F) *T. nuttallii* at Klaskino Inlet, locations 2 and 3 combined. All samples are from subtidal (>2 m depth) locations except the intertidal sample at Seal Island (C).

Island (Fig. 3C and D, Fig. 4C and D). Mean size and age of the four *T. capax* from Ritchie Bay were 148 mm SL and 10.8 y, and the three *T. nuttallii* from the Seal Island subtidal were 186 mm SL and 17.3 y, respectively.

Mortality

Mean mortality rates (with 95% confidence limits) for *T. nuttallii* > 10 y were estimated to be 0.44 (0.26 to 0.63) at Ritchie Bay, 0.20 (0.06 to 0.33) at Klaskino Inlet, and for *T. capax* 0.20 (0.04 to 0.35) at the Seal Island intertidal zone. Since a significant ($P < .05$) relationship between the $\log_e$ of the frequency and age could not be obtained for *T. capax* >10 y from the Seal Island subtidal zone, we estimated mean mortality rates (with 95% confidence limits) for *T. capax* at >3 y [which provided for additional samples on the descending right limb of the age frequency curves (Fig. 4C and D)] to be 0.16 (0.05 to 0.26) for the subtidal and 0.15 (0.09 to 0.21) for the intertidal zones of the Seal Island study area.

Growth

Age–Shell Length

Tresus nuttallii from Klaskino Inlet had similar growth rates to those from Lemmens Inlet, but were higher than those from

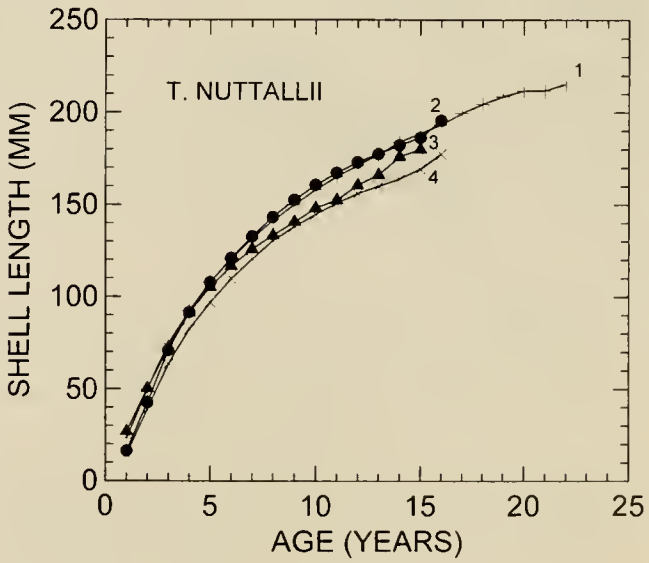

Figure 5. Relationship between mean shell length and age for *T. nuttallii* sampled from: 1. Klaskino Inlet during 1993 (+) (this study); 2. Lemmens Inlet during 1989 (●) (after Campbell et al. 1990); 3. Ritchie Bay during 1993 (▲) (this study); 4. Newcastle Island during 1989 (×) (after Campbell et al. 1990). Equations are presented in Table 1.

TABLE 1.

von Bertalanffy growth parameters for horse clams from British Columbia. Depth zone for subtidal was greater than 2 m, low intertidal was −1 to 1 m, and high intertidal was less than −1 m.

Area	Year	L_∞	k	t_0	Source
T. nuttallii (subtidal)					
Ritchie Bay	1993	200 (±13)	0.139 (±0.024)	−0.15 (±0.33)	This study
Klaskino Inlet	1993	231 (±6)	0.116 (±0.010)	−0.15 (±0.23)	This study
Newcastle Island	1989	183 (±5)	0.168 (±0.012)	0.51 (±0.10)	Campbell et al. (1990)
Lemmens Inlet	1989	202 (±3)	0.167 (±0.006)	0.50 (±0.05)	Campbell et al. (1990)
T. capax (subtidal)					
Seal Island	1993	192 (±5)	0.148 (±0.013)	−0.13 (±0.22)	This study
Lemmens Inlet	1989	195 (±7)	0.154 (±0.016)	−0.01 (±0.18)	This study
T. capax (low intertidal)					
Seal Island	1993	196 (±13)	0.139 (±0.027)	−0.26 (±0.41)	This study
T. capax (high intertidal)					
Kitkatla Inlet	1990	149 (±15)	0.180 (±0.043)	0.17 (±0.29)	Bourne & Cawdell (1992)
Seal Island	1969	155 (±5)	0.189 (±0.021)	−0.11 (±0.23)	Bourne & Smith (1972)
Doyle Island	1971	169 (±4)	0.132 (±0.008)	−0.10 (±0.13)	Bourne & Smith (1972)

Values in brackets are approximate 95% confidence intervals.

Ritchie Bay and Newcastle Island (Campbell et al. 1990) (Fig. 5, Table 1). There were no differences in growth rates between *T. capax* collected between the low intertidal and subtidal zones from Seal Island (Table 1) so data were combined for graphical purposes (Fig. 6). Growth rates were similar for *T. capax* from Seal Island and Lemmens Inlet (Fig. 6, Table 1). Growth was more rapid for *T. capax* in the low intertidal and subtidal areas studies than for those sampled in the high intertidal areas in other studies in B.C. (Fig. 6, Table 1).

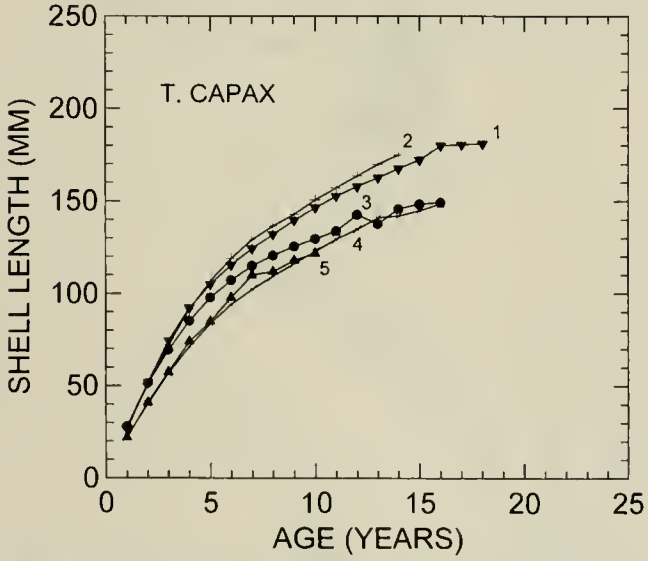

Figure 6. Relationship between shell length and age for *T. capax* sampled from: 1. Seal Island during 1993, low intertidal and subtidal combined (▼) (this study); 2. Lemmens Inlet subtidal zone during 1989 (+) (this study); 3. Seal Island during 1969 (●) (after Bourne and Smith 1972b); 4. Doyle Island during 1971 (×) (after Bourne and Smith 1972b); 5. Kitkatla Inlet during 1990 (▲) (after Bourne and Cawdell 1992). Areas 3, 4, and 5 are from the high intertidal beach zone. Equations are presented in Table 1.

Length–Weight

All length–weight relationships were positively and highly correlated, indicating that weights increased with SL increases (Table 2, Figs. 6 and 7). There were no significant differences (ANCOVA, $P > .05$) in slopes or elevations for all length–weight relationships between *T. capax* from the low intertidal and those from the subtidal in the Seal Island study area, so the data for each depth zone were combined. For total wet weight and whole drained wet weight and SL relations, there were no differences in slopes between all areas and although there were no differences in elevations between Lemmens Inlet and Seal Island for *T. capax*, weights were significantly higher for *T. nuttallii* from Klaskino Inlet and significantly lower for *T. nuttallii* from Ritchie Bay than for *T. capax* from the other two locations (ANCOVA, $P < .01$) (Table 2, Figs. 6 and 7). The shell weight-SL relations were similar for horse clams from Klaskino and Lemmens Inlets and Seal Island, however they were heavier at all three sites compared to those from Ritchie Bay. Although body weights were heavier than shell weights at all SL for *T. capax* at both Lemmens Inlet and Seal Island (Fig. 8C and D), shell weights became heavier than body weights for *T. nuttallii* at about SL >170 mm for Ritchie Bay and SL > 150 mm for Klaskino Inlet (Fig. 8A and B). Neck weights grew less than the other body parts studied and were similar for the three areas sampled (Table 2, Fig. 8).

Age–Weight

All the age–weight relationships were positively and significantly correlated, indicating that weights increased with increasing age (Table 2). Although the age–weight relationships showed similar trends to the weight–RL relationships there were considerably more variation in the R^2 and inter-area comparisons in the former than the latter (Table 2).

Mean Weights and Biomass

Mean total wet weight and drained total weight (g) (±1 SE in brackets) of horse clams was 671.4 (9.3) and 493.7 (7.0) (n = 507)

TABLE 2.

Regression coefficients for different morphological relationships of *T. nuttallii* from (1) Ritchie Bay and (2) Klaskino Inlet and *T. capax* from (3) Lemmens Inlet and (4) Seal Island for equation $\log_e Y = \log_e A + B \log_e X$, where X is the shell length (Sl in mm) or age (y) and Y variables are weights (g).

Variables			Regression Coefficients			
Y	X	Area	A	B	R^2	*n*
Total	SL	1	−8.980	3.053[a]	.913	90
		2	−9.442	3.185[a]	.893	295
		3	−9.865[a]	3.290[a]	.955	73
		4	−8.858[a]	3.092[a]	.989	124
Drained	SL	1	−10.115	3.213[a]	.964	90
		2	−9.743	3.185[a]	.893	295
		3	−10.167[a]	3.289[a]	.912	73
		4	−9.125[a]	3.084[a]	.979	124
Body	SL	1	−8.330	2.729[a]	.934	90
		2	−8.067	2.709[a]	.720	295
		3	−9.601[a]	3.063[b]	.906	73
		4	−9.337[a]	3.020[b]	.976	111
Neck	SL	1	−8.381[a]	2.594[a]	.884	90
		3	−10.407[b]	3.006[b]	.881	73
		4	−8.963[ab]	2.731[ab]	.955	111
Shell	SL	1	−13.759	3.786[a]	.963	90
		2	−12.563[ab]	3.608[a]	.886	295
		3	−12.772[a]	3.638[a]	.907	73
		4	−11.840[b]	3.459[a]	.974	111
Drained	Age	1	2.587[a]	1.424[ab]	.858	66
		2	2.685	1.486[a]	.771	332
		3	2.163	1.851	.908	70
		4	2.922[a]	1.306[b]	.930	99
Body	Age	1	2.429	1.225[a]	.838	66
		2	2.698[a]	1.193[a]	.567	291
		3	1.889	1.218[b]	.894	70
		4	2.568[a]	1.225[ab]	.912	99
Neck	Age	1	1.966	1.112[a]	.799	66
		3	0.941	1.643	.823	70
		4	1.820	1.101[a]	.880	99
Shell	Age	1	1.224	1.671[a]	.852	66
		2	1.396	1.728[a]	.792	291
		3	0.859	2.051	.909	70
		4	1.773	1.418	.939	99

Total is whole wet weight, Drained is whole drained total wet weight, Body includes all soft body parts. Minimum and maximum sizes and ages used for equataions, respectively, were 75–196 mm SL and 3–20 y, for Ritchie Bay, 110–229 mm SL and 6–24 y for Klaskino Inlet, 51–169 mm SL and 2–13 y for Lemmens Inlet, and 11–183 mm SL and 1–21 y for the Seal Island. All R^2 values are significant at $P < .01$. Neck weights for Klaskino Inlet were not measured. Coefficients, within the same X and Y combination and in the same column, that are followed by the same letter are not significantly different (ANCOVA, $P > .05$), those not followed by the same letter are significantly different (ANCOVA, $P < .05$).

for Ritchie Bay, 1,190.2 (24.8) and 880.7 (18.4) (n = 341) for Klaskino Inlet, 459.5 (13.1) and 338.3 (9.6) (n = 525) for the low intertidal, and 355.0 (10.3) and 261.6 (7.6) (n = 599) for the Seal Island subtidal area, respectively.

Mean biomass (g/m^2) for *T. nuttallii* was 157.5 for all depths at the Ritchie Bay study area, and for *T. capax* was 2,756.6 for the low intertidal zone and 130.7 for the subtidal zone in the Seal Island study area.

DISCUSSION

Mean densities and biomass were greater for *T. capax* in the low intertidal zone than in the subtidal area at Seal Island and for *T. nuttallii* in the subtidal at Ritchie Bay. Results confirm that *T. capax* was generally found to be more abundant in shallow waters than deeper waters in some locations, and although *T. nuttallii* may be found intertidally (Haderlie and Abbott 1980), *T. nuttallii* was most abundant subtidally. Bourne and Cawdell (1992) and Bourne et al. (1994) found horse clams sampled from intertidal sites in northern B.C. were all *T. capax*. The relative abundance in subtidal zones between *T. capax* and *T. nuttallii* can vary considerably between locations: in some areas one species may be overwhelmingly more abundant than the other (e.g. >99% of *T. nuttallii* at Ritchie Bay and Klaskino Inlet compared to >99% of *T. capax* at Sandy Islets) or there may be a considerable species mix (e.g., 78.3% *T. nuttallii* and 21.7% *T. capax* from a commercial sample (n = 783) at 5–9 m depths at Lemmens Inlet during 1989, A.

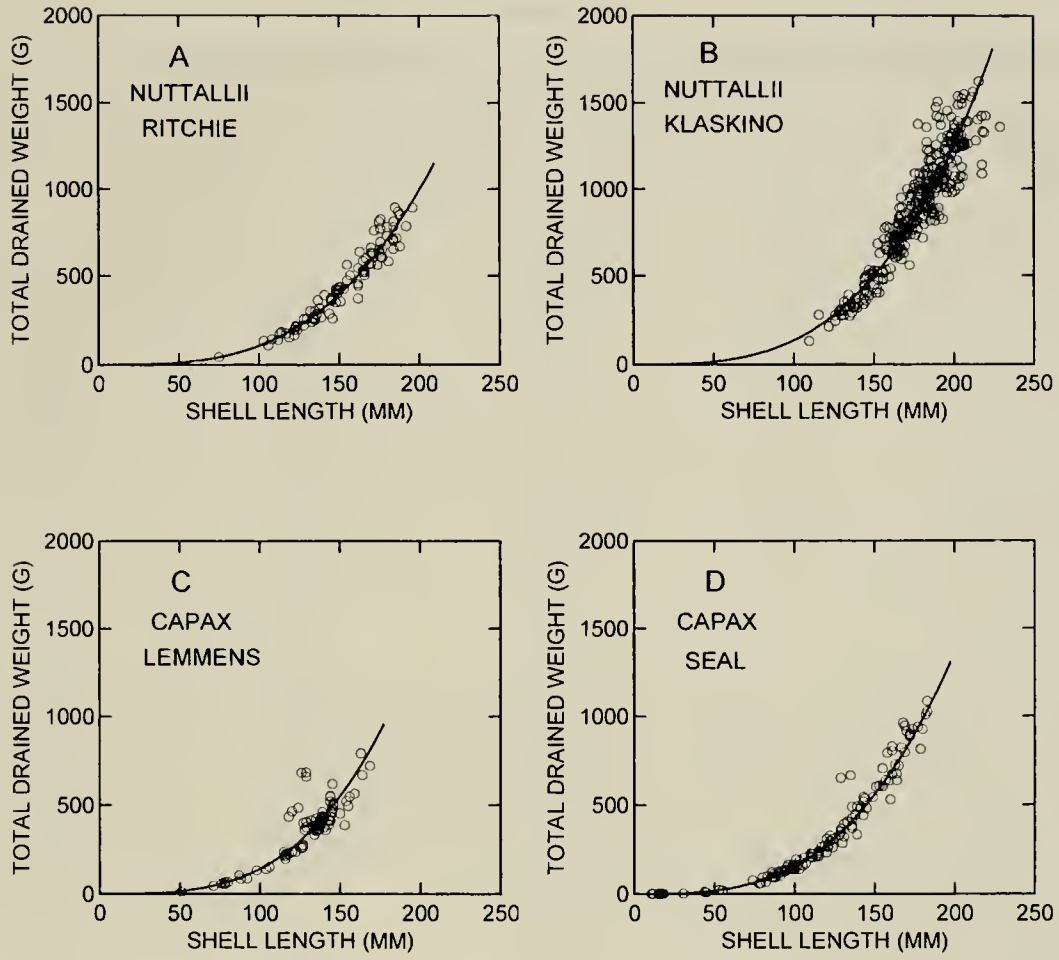

Figure 7. Total drained and shell length relationship for *T. nuttallii* collected from (A) Ritchie Bay, and (B) Klaskino Inlet, and *T. capax* from (C) Lemmens Inlet, and (D) Seal Island (low intertidal and subtidal samples combined). Equations are presented in Table 2.

Campbell, unpublished data) (Stout 1967, Wendell et al. 1976). Distribution and density of both species have been found to vary considerably and their distribution was often aggregated (e.g., Stout 1967, Wendell et al. 1976, Goodwin and Shaul 1978).

The large mean sizes and ages of *T. nuttallii* sampled from Ritchie Bay and Klaskino Inlet were probably a result of an accumulation of older individuals in an unharvested population, with probably little or no recruitment having occurred within 5 y prior to sampling. In contrast, the age frequencies of *T. capax* from Seal Island were dominated by large numbers of 4–6 y individuals. There may have been many more small individuals <3 y, which could not be monitored by the sampling method used, consequently this study could not examine settlement, abundance and mortality of horse clams in their first few years of life. Wendell et al. (1976) found that recruitment of recently settled horse clams varied spatially and annually between beds.

Mean natural mortality rate estimates for adult horse clams were between 0.15 and 0.44 depending on location and species examined. These mortality values are within the values predicted with Hoenig's (1983) generalized mortality equation (0.36 for a max age of 15 y, 0.26 for max age of 22 y, and 0.24 for a max age of 24 y). Wendell et al. (1976) suggested a theoretical maximum age of over 20 y and an average longevity of 15 y for *T. capax* in Humboldt Bay, California. Wendell et al. (1976) found that mortality of young of year recruits was sufficient to inhibit successful recruitment for 2 y in some Humboldt Bay *T. capax* populations. Natural mortality in *Tresus* juveniles and adults may be caused by haplosporidian parasites (Armstrong and Armstrong 1974), invertebrate predators (e.g., sea stars, *Pisaster brevispinus* Stimpson 1857, moon snails, *Polinices lewisii* Gould 1847, crabs such as *Cancer magister* Dana 1852 (Wendell et al. 1976, Sloan and Robinson 1983), fish (Stout 1967, Laurent 1971) and sea otters, *Enhydra lutris* Merriam 1923 (Kvitek et al. 1988, Kvitek and Oliver 1992, Watson and Smith 1996).

Growth rates for *T. capax* in the low intertidal and subtidal areas reported in this paper were faster than those reported for other mid intertidal areas in B.C. (Bourne and Smith 1972b, Bourne and Cawdell 1992). Breed–Willeke and Hancock (1980) showed that *T. capax* from subtidal regions grew more rapidly than those from intertidal areas in Yaquina Bay, Oregon. Wendell et al (1976) showed intertidal and subtidal *T. capax* growth rates to differ significantly between beds and between year classes within a bed in Humboldt Bay. There was considerable variation in growth between populations of *T. nuttallii* (this study, MacGinitie 1935, Marriage 1954, Laurent 1971, Clark 1973, Campbell et al. 1990). In this study, with increasing size, shells became heavier

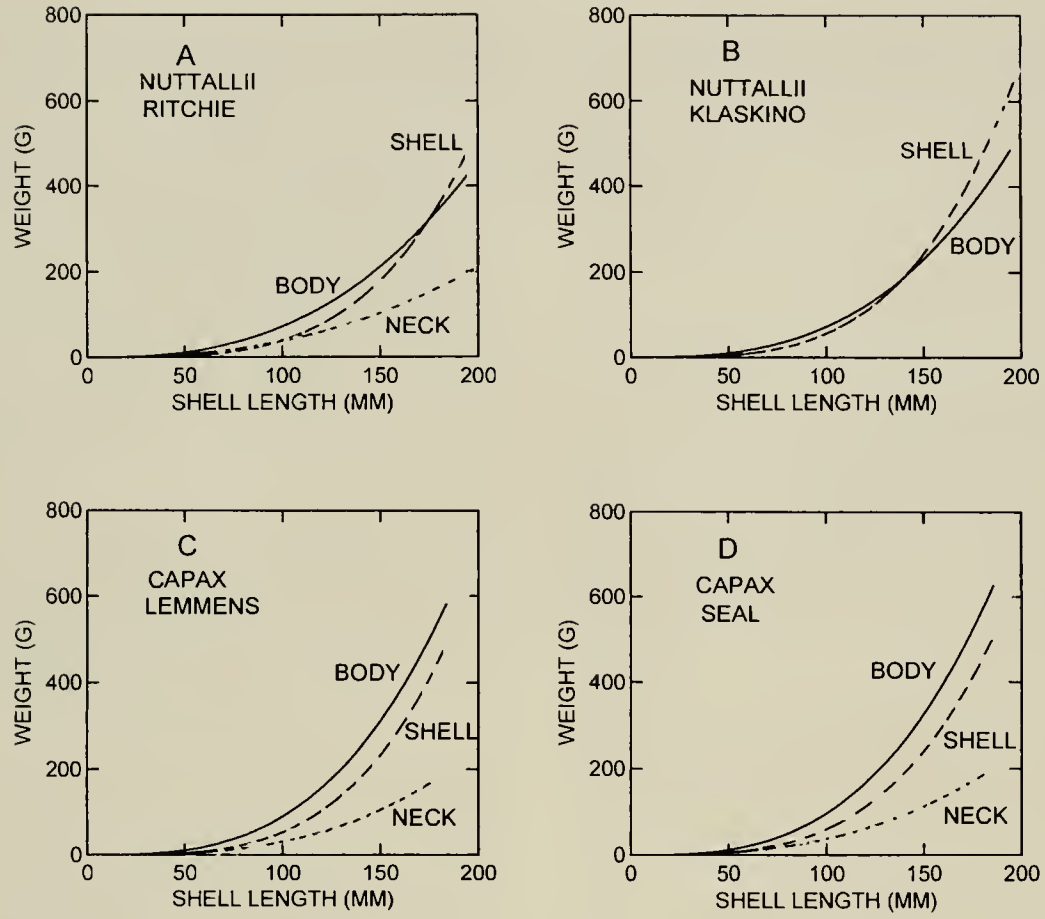

Figure 8. Body, shell, and neck weight and shell length relationships *T. nuttallii* collected from (A) Ritchie Bay, and (B) Klaskino Inlet, and *T. capax* from (C) Lemmens Inlet, and (D) Seal Island (low intertidal and subtidal samples combined). Equations are presented in Table 2.

than the soft body parts for *T. nuttallii* compared to *T. capax*. The reasons for the differences in growth rates in *T. nuttallii* and *T. capax* are unknown, but may be attributed to differences in a variety of environmental factors associated with habitat, such as food availability, temperature, current patterns, and substrate types.

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SIZE AND AGE AT SEXUAL MATURITY AND SEX RATIO IN OCEAN QUAHOG, *ARCTICA ISLANDICA* (LINNAEUS, 1767), OFF NORTHWEST ICELAND

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ABSTRACT Ocean quahogs, *Arctica islandica*, were collected in February 1994 from near-shore populations off north-west Iceland for determination of developmental stages relative to size, age, and sex. A microscopic examination of histologically prepared tissue of 206 ocean quahogs showed that *s6* specimens (31–70 mm shell length) were immature and could not be sexed. Sexual differentiation was evident in 200 individuals, 17 were in the intermediate stage and 183 were mature. On the basis of annual internal growth bandings in the shells of the specimens, the age ranged between 7 and 32 y in the intermediate stage and the individuals were from 24 to 60 mm in shell length. The smallest and youngest individuals that could be sexed were males. The smallest mature male was 36 mm in length, but the youngest aged individual was 10-year-old (49 mm length). The youngest mature female was 44 mm and 13-year-old. Age and size of maturity showed a wide range and may be dependent on growth rate and environmental conditions. Sex ratio between males and females was examined relative to size in 200 quahogs. The male to female (M:F) ratio varied between size classes, with males dominating in the smallest size classes, which may be related to their earlier development of germinal cells. After 40 mm length was reached, the females dominated in all size classes except 65 to 69 mm (1.3:1) and 70 to 74 mm (1:1).

KEY WORDS: *Arctica islandica*, ocean quahog, sexual maturity, sex ratio, Iceland.

INTRODUCTION

The ocean quahog, *Arctica islandica*, is widely distributed over the continental shelves of both Europe and North America. In Icelandic waters there have been reported great densities of commercial potential (Eiríksson 1988, Thorarinsdóttir & Einarsson 1996).

The ocean quahog has been the focus of an important commercial fisheries in the United States since 1976 (Murawski & Serchuk 1989) and with growing demand for this species an interest has arisen in Iceland to investigate the possibility for developing an Icelandic fishery for human consumption. As a result of this interest a study was undertaken in 1994 to assess the distribution, abundance, and biology of this species in Icelandic waters (Thorarinsdóttir & Einarsson 1996, Thorarinsdóttir & Jóhannesson 1996). The examination of size and age of sexual maturity and sex ratio is reported on in this paper.

Studies on size and age at sexual maturity in ocean quahog have been undertaken in the Mid-Atlantic Bight area of the United States (Thompson et al. 1980b, Ropes et al. 1984) and in Nova Scotia, Canada (Rowell et al. 1990), but such studies have not been made in ocean quahog from Icelandic waters before.

MATERIALS AND METHODS

Specimens of *A. islandica* were dredged off the northwest coast of Iceland during a shellfish assessment survey in February 1994. In each of 7 locations visited during the survey, about 30 specimens were sampled for the study of shell length, age, and sexual maturity (Fig. 1). The size-frequency distributions of *A. islandica* captured by sampling dredges are normally dominated by large individuals with small shells being rare (Fogarty 1981, Murawski et al. 1982, Thorarinsdóttir & Einarsson 1996). In this study few specimens smaller than 40 mm in shell length were collected by the hydraulic dredge. Out of the 206 individuals investigated in the present study, only 10 had a shell length less than 40 mm.

The samples from the various sampling sites were pooled together and returned to the laboratory where a piece of the gonads 4- to 5-mm wide was cut from the hinge region to the ventral region of the mantle edge. In small animals sections included the whole gonads. The gonads were preserved in 10% formaldehyde in seawater. Histological preparation of the gonads included embedding in paraffin, sectioning at 8 μm, and staining with haematoxylin and eosin. The stained preparations were examined microscopically for the presence of differentiated gametes.

Those specimens having little follicular development, no cellular structures definable as male or female, and much of the gonad filled with connective tissue were designated as undifferentiated. Those with sufficient development to be differentiable as males or females were further classified as intermediate or mature in their gonadal development according to the criteria used by Rowell et al. (1990). Intermediate specimens were typified by reduced-to-sparse follicular development with follicles widely spaced and separated by vesicular connective tissue. The follicles themselves displayed varying degrees of development, from those with small diameter and lacking germinal cells in portions of the epithelium to those typifying the mature condition with large diameter, little connective tissue, and a completely filled the gonadal area.

The height of the shells was measured with vernier calipers to the nearest millimeter. For the purpose of comparison with other investigations dealing with size and age at sexual maturity, the shell height estimates in the present study were converted to shell length using a ratio height:length of 0.91 (Witbaard 1997).

Sex ratio of the clams was examined relative to size in 5-mm size classes for a total of 200 animals having shell-lengths ranging from 24 to 119 mm.

The age data was reached by examining acetate peels of a cross-section of the left valve of each individual and by counting the growth lines in the hinge tooth (Ropes 1987). The growth lines in the hinge tooth have been shown to correspond in number to those in the valve itself (Ropes et al. 1984, Thompson et al. 1980a, Thompson et al. 1980b).

RESULTS

Of the 206 quahogs studied, 6 ranging in length from 31 to 70 mm were found to be sexually undifferentiated. Only 2 of these

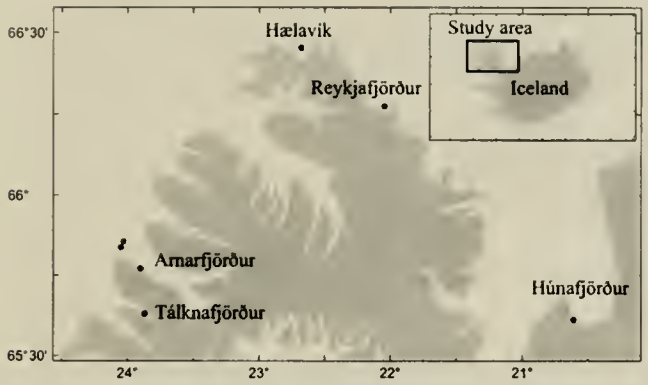

Figure 1. Sampling sites of *A. islandica* off northwest Iceland used for determination of size and age for sexual maturity.

individuals, 31 and 70 mm in length were aged and had the age of 6 and 43 y, respectively (Table I). The gonia were embedded in the germinal epithelium and lacked definable cellular features. Gonadal follicles were sparse, of small diameter, and were surrounded by an abundant loose vesicular connective tissue. The lumen of the follicles were empty (Fig 2a).

Sex could be determined in 200 specimens; 92 males and 108 females. Seventeen quahogs, 7 males and 10 females were determined to be intermediate in their gonadal development. The males ranged in length from 24 to 60 mm, with a mean of 44 mm. Of these, 2 animals 34 and 45 mm in length were aged 7- and 9-year-old, respectively. Intermediate females ranged from 35 to 58 mm in length. Of these, 1 52-mm female was aged 32-year-old (Table I). For both sexes the follicles were of small diameter and sparsely scattered among a loose vesicular connective tissue. The lumina of the follicles were often free of cells. Females were distinguishable from males by the presence of enlarging oocytes and some spermatogenesis could bee seen in the males (Fig. 2, b and c). There were 183 mature quahogs found; 84 males and 99 females. Males ranged in the length from 36 to 103 mm with a mean of 75 mm, while females ranged from 44 to 119 mm in length with a mean of 74 mm. Of those that could be aged there were 114 mature specimens; 53 males and 61 females. Mature males with lengths of 49 to 99 mm ranged from 10 to 108 y with a mean of 50 y. Mature females with lengths of 44 to 104 mm ranged from 13 to 120 y with a mean age of 53 y (Table I). In these quahogs the follicles were greatly expanded and often filled the gonadal area, with little connective tissue occurring between adjacent follicles. In females the oocytes crowded into the lumen of follicles. The diameter of fully ripe oocytes that were measured ranged from 54.2 to 74.1 μm and the average was 63.9 μm (Fig. 2d). In males, spermatocytes and spermatids proliferated from the germinal epithelium and sperm predominated the lumen of the follicles (Fig. 2e). However, the intermediate and mature specimens displayed all phases of the gametogenic cycle between early active and partially spawned, and the same individual could show more than one phase. The 91 males and 109 females studied yielded a male:female ratio of 0.8 (Table 2). No evidence of hermaphroditism was observed. When males and females were pooled by 5-mm-length categories, the males dominated in the smallest size categories, as no female was identified smaller than 35 mm in length. With increased shell length, females dominated and no males were observed having shell length greater than 103 mm. The largest female was 119 mm long.

DISCUSSION

Investigations made on *A. islandica* have shown a continuous annual gonadal cycle (Loosanoff 1953, Jones 1981, Mann 1982, von Obertzen1972, Rowell et al. 1990) with well-defined sex cells present in the follicles throughout the year, even immediately following spawning. This makes it possible to estimate age and size at sexual maturity in samples collected at any time of the year.

In the present study male ocean quahogs seem to begin producing germinal cells at a smaller size and probably younger age than females, as reported for the eastern coast of the United States and Canada (Ropes et al. 1984, Rowell et al. 1990).

The minimum age and size of maturation from undifferentiated to intermediate stage and from intermediate to mature stages was very variable. The variability observed might be attributed to differences in growth rate due to different environmental conditions, as the longest distance between sampling sites was about 100 km (Fig. 1). However, Rowell et al. (1990) had similar results for ocean quahogs from a single locality, suggesting that local conditions are less important than suggested for this species before (Thompson et al. 1980). The explanation of the variability observed might be variability inherent to the individuals making up the population. Dahlgren et al. (2000) assessed genetic subdivision in *A. islandica* from the North American coastline, Icelandic waters, Faroe Islands, Norway, and Sweden. The greatest diversity (haplotypes per individual sampled) was found in the population from Iceland.

All undifferentiated or immature individuals observed in the

TABLE 1.

Stages of gonadal development relative to shell length, age, and sex.

	Undifferentiated		Intermediate				Mature			
			Males		Females		Males		Females	
	Total	Aged	Total	Aged	Total	Aged	Total	Aged	Total	Aged
Number	6	2	7	2	10	1	84	53	99	61
Length (mm)	31–70	31, 70	24–60	34, 45	35–58	52	36–103	49–99	44–119	44–104
X Length	43	—	44	—	46	—	75	73	74	74
SD Length	14	—	14	—	7	—	10	10	14	13
Age (y)	—	6, 43	—	7, 9	—	32	—	10–108	—	13–120
X Age	—	—	—	—	—	—	—	50	—	43
SD Age	—	—	—	—	—	—	—	20	—	26

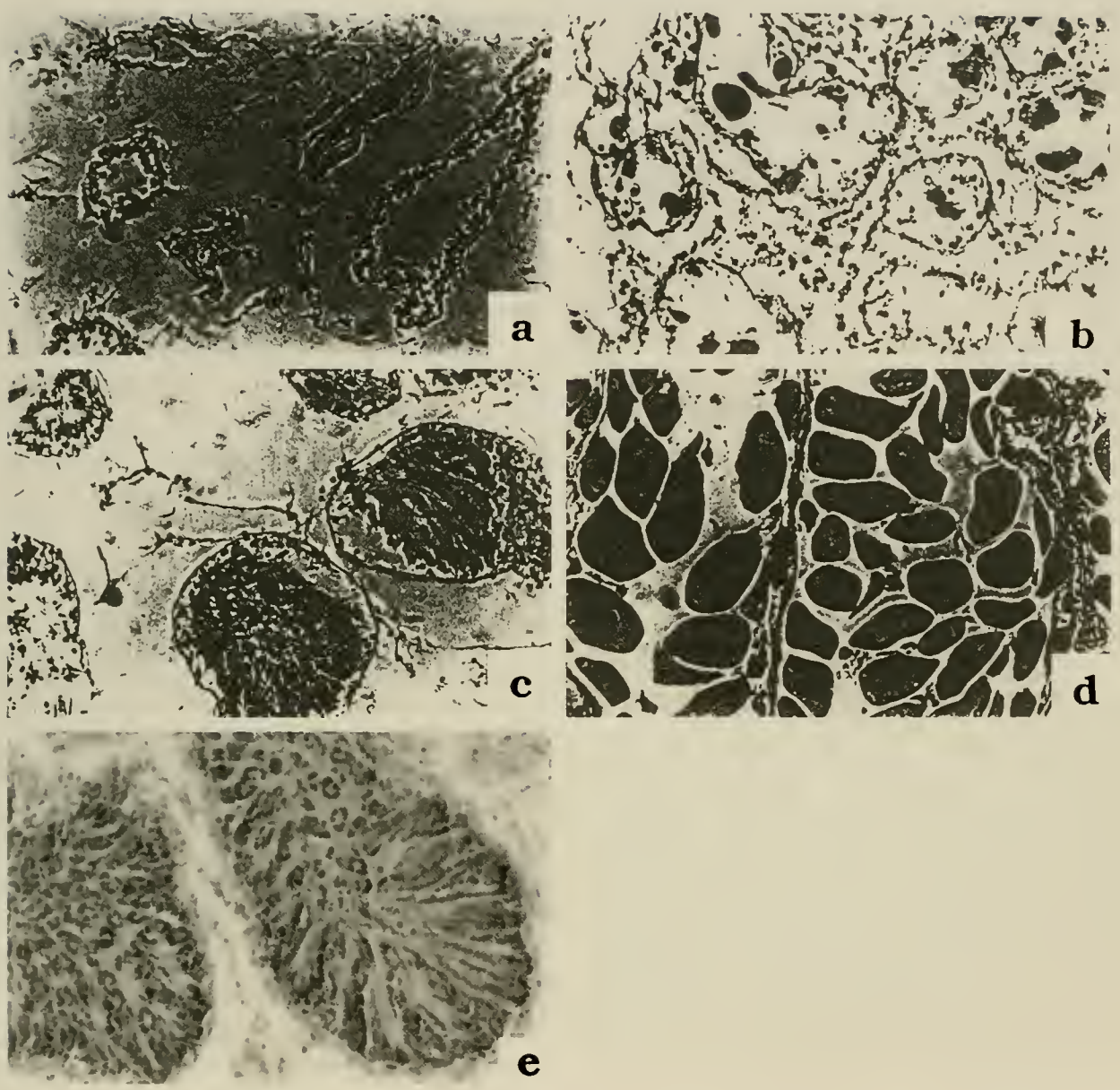

Figure 2. Photomicrographs (×30) of transverse sections through gonads of *A. islandica* at various stages in gamete development. (a) Undifferentiated gonadal tissue from a 6-year-old individual 31 mm in shell length. (b) Intermediate gonadal tissue from a 32-year-old female, 52 mm in shell length. (c) Intermediate gonadal tissue from a 9-year-old male, 45 mm in shell length. (d) Fully mature gonadal tissue from a 45-year-old female, 64 mm in shell length. (e) Fully mature gonadal tissue from a 44-year-old male, 62 mm in shell length.

present study had shell length less than 61 mm except 1 immature individual of shell length 70 mm and 43 years of age. It is difficult to explain the immaturity of this large and old individual, but it might indicate that some individuals do not reach maturity at all. The observed size and age at sexual maturity is somewhat higher than reported from studies on quahogs off the east coast of North America. However, it is difficult to compare the age of the immature individuals in the present study to the age observed off the east coast of America, as only 2 specimens were aged and 1 of them seems to be exceptional both in age and size. Immature ocean quahogs were observed to a maximum length of 47 mm and 14 years of age off Rhode Island (Thompson et al. 1980b) and to 46 mm length and 8 years of age off Long Island (Ropes et al. 1984). From coastal waters in Nova Scotia, the maximum shell length and age for immature individuals was 45 mm and 12 y, respectively (Rowell et al. 1990).

The size range of ocean quahogs entering the intermediate stage of maturity in the present study was 24 to 60 mm for males and 35 to 58 mm for females with the mean size of 44 and 46 mm, respectively. Only 3 of 17 individuals were aged and the age ranged between 7 and 32 y. For ocean quahogs from Canada this size has been found to be somewhat lower, or 21 to 48 mm for males and 25 to 53 mm for females with the mean length being 30 and 34 mm, respectively. The age in these individuals ranged from 3–24 y (Rowell et al. 1990). Off the east coast of the United States the size range for males from Long Island was 21–48 mm and 36–45 mm for females and the age ranged from 3 to 10 y for the males and 5 to 8 y for the females (Ropes et al. 1984). Thompson

TABLE 2.

Male:female sex ratio relative to length.

Length (mm)	Numbers		M:F Ratio
	Males	Females	
20–24	1	0	—
30–34	2	0	—
35–39	1	1	1:1
40–44	2	4	0.5:1
45–49	2	4	0.5:1
50–54	3	3	1:1
55–59	3	12	0.3:1
60–64	6	10	0.6:1
65–69	22	17	1.3:1
70–74	14	14	1:1
75–79	15	17	0.9:1
80–84	7	8	0.9:1
85–89	7	9	0.8:1
90–94	2	3	0.7:1
95–99	3	4	0.7:1
100–104	1	2	0.5:1
105–109	0	1	—
115–119	0	1	—
Total	91	109	0.8:1

et al. (1980b) found intermediate-stage individuals from 25 to 50 mm length and 6- to 15-year-old off Long Island.

In the present study a male as small as 36 mm was considered sexually mature. The smallest mature male was aged 10-year-old and 49 mm in shell length and females aged 13-year-old and 44 mm were considered mature. This size and age at maturity is similar to records from the eastern coast of the United States. Furthermore, Thompson et al. (1980b) reported the smallest mature male ocean quahog to be 42 mm and 11-year-old and the smallest female 50 mm and 11-year-old. Ropes et al. (1984), however, reported 36 mm and 5 y for males and 41 mm and 6 y for females. Rowell et al. (1990) found the smallest mature quahog in Nova Scotia to be of 27 and 30 mm length for males and females, respectively. The smallest aged mature individuals were of 7 y and 40 mm for both sexes.

For the 200 individuals in the intermediate and mature stage of gonadal development, all phases of the gametogenic cycle were displayed and some were approaching ripeness or were ripe. The main spawning time for *A. islandica* in Icelandic waters is from June to August, but the spawning is protracted (Gudrun Thorarinsdóttir 2000), as previous studies off the United States and Canada have also indicated (Mann 1982, Rowell et al. 1990).

The life span of *A. islandica* is very long. Individuals over 100 years of age are common and the oldest individual from Icelandic waters was 202-year-old (Steingrímsson & Thorarinsdóttir 1995). The development of the continuous reproductive potential in *A. islandica* after the age of 7 to 32 years and the length of 24 to 60 mm seems consistent with the estimates of the species long life span. Continuous reproduction during a long life span can be beneficial for *A. islandica* and may be an evolutionary strategy in response to uncertain larval and juvenile survival. Thompson et al. (1980b) conclude that there is no obvious indication of senility for *A. islandica* of 100 or more y in regards to spawning. However, in the present study the gonads of the oldest individuals contained smaller follicles than the younger mature individuals, possibly indicating senility.

The sex ratio showed predominance of males in the smallest size classes, which may be explained by the smaller size at which males reach the intermediate stage or it may be due to small numbers of individuals in these classes. The females were dominating in all the size classes bigger than 40 mm in shell length except in the 65 to 69 mm size class. Previous studies of sex ratios in ocean quahogs have generally indicated ratios in favor of males (Jones 1981, Ropes et al. 1984, Rowell et al. 1990), although results have been quite variable. The hypothesis that female ocean quahogs may live longer than males based on predicted ages of ocean quahogs at a marking site (Murawski et al. 1982) and observed shift in sex ratio from male to female in the highest age classes (Fritz 1991, Ropes et al. 1984, Rowell et al. 1990) has some support in the present study, but due to the small sample number in the bigger size classes it can not be ascertained.

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GROWTH AND REPRODUCTIVE PATTERNS IN *VENERUPIS PULLASTRA* SEED REARED IN WASTEWATER EFFLUENT FROM A FISH FARM IN GALICIA (N.W. SPAIN)

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ABSTRACT The use of effluent water from a turbot (*Psetta maxima*) farm for culturing *Venerupis pullastra* juveniles (seed) was evaluated. Reproductive activity, growth, condition, and survival rate were investigated. After a 2-mo acclimation period, clam seed (mean live weight = 360 ± 18 mg SD, mean length = 14 ± 1.7 mm SD) were grown from June 30 to November 20 of 1993 in three different flow-rates of wastewater. Thirty individuals were sampled from each tank biweekly. Stability in the main environmental parameters of the effluent (temperature, salinity, oxygen, etc.) provided good conditions for seed growth. After the acclimation period, mortality was less than 18%. Growth-rate coefficients (K values) of *V. pullastra* were correlated positively with increase in the effluent flow and were significantly higher than in the control tanks (K = .001). Shell length (27.4 ± 1.8 mm SD) and, in particular, live weight (2,907.6 ± 530.9 mg SD) were highest in tanks with a flow of 4 vol/h and these tanks also produced the fastest growth rate (K = .0445). In tanks with flows of 1 vol/h and 2 vol/h, final weights and lengths were 1,823 ± 273.2 mg SD, 23 ± 1.8 mm SD and 2,361 ± 351.4 mg SD, and 25 ± 1.4 mm SD, respectively. Condition indices of clams followed a similar pattern reaching final values ranging from 18 to 28 and were significantly higher than in the control tanks. High condition index values might be due mostly to the high amount of food present in the effluent water throughout the experiment. Stereological and histological techniques were used to determine gametogenic activity. Successive spawnings and recovery of the gonad, which are characteristic of this species, were noted in clams in experimental tanks. However, they occurred in a shorter time period. Results suggest that it is possible to rear *V. pullastra* seed under these special culture conditions and that a better balance between volume of effluent used and clam productivity was achieved with a flow rate of 2 vol/h.

KEY WORDS: clam, effluent water, gametogenic cycle, growth, seed, *Venerupis pullastra*

INTRODUCTION

Galicia (N.W. Spain) is among the greatest consumers of clams in the world. Two species are especially popular in Galicia. In order of economic importance, these are *Ruditapes decussatus* and *Venerupis pullastra*. The two species have a similar market value, but *V. pullastra* has the advantage of a faster growth rate (Pérez-Camacho and Cuña 1987), although it is less resistant to tidal exposure and low salinities. Depletion of natural and introduced stocks of clams has caused an increased demand for hatchery-produced seed (Laing et al. 1987, Beiras et al 1993), but the large amount of food (mainly microalgae) needed to culture postlarval bivalves for extensive periods beyond settlement makes nursery operations too costly.

The largest turbot (*Psetta maxima*) culture industry in the European Community (1300 ton/year, Unidade Estadística, Xunta de Galicia, personal communication) is located in Galicia. This industry discards daily considerable quantities of untreated to sea water to the sea. Only 20–30% of the nitrogen and phosphorus added as food to the marine fish ponds is consumed by the fish (Krom et al. 1985a, Krom et al. 1985b, Porter et al. 1987). There is a considerable literature describing the biological treatment of such effluent by using it to grow algae (Ryther et al. 1972, Ryther et al. 1975, Cohen and Neori 1991, Shpigel et al. 1993b) or to feed bivalves (Gordin et al. 1981, Shpigel and Fridman 1990, Trevor and Iwama 1991, Shpigel et al. 1993a, Shpigel et al. 1993b). The main purpose of those studies was to improve the quality of the effluent and minimize its effect on the environment (Krom et al. 1985a, Krom et al. 1985b, Porter et al. 1987, Shpigel and Blaylock 1991). It is possible that effluent from intense turbot culture operations, with high levels of nutrients, could be used as a food source for clam seeds, thus reducing costs of the bivalve nursery and reducing the negative effects of the effluent on the environment. We have already successfully grown *Ruditapes decussatus* seed in effluent from turbot ponds (Jara-Jara et al. 1997).

In this study, we investigated the possibility of rearing *Venerupis pullastra* seed in effluent from turbot culture operations. Acclimation period, growth rate, gonad development and survival rate of *V. pullastra* were investigated.

MATERIALS AND METHODS

Experimental Procedure

The experiment was carried out on a pilot-scale level at a private turbot farm at Nastos, from April 29 to October 20 1993. Hatchery-reared *Venerupis pullastra* seed with a live weight of 9 ± 0.8 g SD and length 2.7 ± 0.7 mm SD was maintained for an acclimation period in an open flow system at a density of 0.1 kg/m^2 in two rectangular fiber glass tanks (4.0 × 1.4 0.4 m) with a sand substrate. Flow rate of the effluent from the turbot culture operation was sufficient to replace the water volume of each tank twice every hour.

After an acclimation period of approximately 60 days, the seed (mean live weight 360 ± 18 mg SD and about 14 mm shell length), was placed in 12 rectangular tanks (4.0 × 1.4 × 0.4 m deep) with a total water volume of about 560 L. The bottom was covered with 10 cm sand. The initial density was 0.5 kg/m^2. Different flow rates were used to determine the effect of the flow rate on growth and to manage the rate of use effluent water effectively. Three flow rates replicated 3× were tested. In three sets of tanks, the wastewater flow rate was such to produce one, two and four complete water exchanges per hour (560, 1,120, and 2,240 L/h, respec-

tively). A fourth set of tanks received only fresh sea water at a rate of two tank vol/h, and served as control. For the remainder of this article, flow rates in tanks will be expressed as 1 × h, 2 × h, and 4 × h.

Biweekly between June 30 and November 20 1993, 30 individuals from each tank (90 clams per flow rate) were sampled randomly and placed in filtered sea water at 17 °C for 24 h. Total shell length and height of each individual were measured to the nearest 0.1 mm with vernier callipers and after drying each clam on absorbent paper for 10 min to remove surface water, total weight of each clam was measured to the nearest 0.1 g using an electronic balance (±0.1 mg). Soft parts were separated from the shell and both were dried to constant weight at 80 °C. Condition index (CI) was calculated by: CI = (mean dry meat weight/mean dry shell weight) × 100.

Relative daily growth rates (K) were calculated using the equation:

$$K = (\ln W_2 - \ln W_1)/t_2 - t_1$$

where: W_1 = the initial mean total weight, W_2 = the final mean total weight, $t_2 - t_1$ = the elapsed time in days. The coefficient K, multiplied by 100, yields the % change per day.

Statistical analysis of the results was performed using a one-way ANOVA and the differences were tested by Duncan's multiple range test.

Water Quality

Water temperature, salinity, and oxygen concentration were measured daily (from April to November 1993) in the experimental tanks. Temperature was measured with a thermometer (±0.1 °C), salinity with an ATGO model S/MILL salino-meter, and oxygen level was always maintained above 7–8 mg/L by injecting liquid oxygen constantly into the turbot culture tanks. Chlorophyll *a* concentration of effluent from turbot culture ponds was determined every 15 days from April 1993 to October 1993. One- to three-liter aliquots were concentrated on GF/C filters and chlorophyll *a* was extracted with 90% acetone and assayed according to the Strickland and Parsons (1972) method.

The turbot were fed daily with a pelletized diet (EWOS turbot 4.5 mm and 12% humidity) containing 49% protein, 14% lipid, 5% carbohydrate, 12% ash, 1.5% fiber, 6% vitamin premix, 2% calcium, 1% phosphorus, 0.2% sodium, with a digestible energy of the 17.4 Kj/g. Relative values (kg of component/tonne of fish) of organic particulate matter, total nitrogen, and phosphorus in the effluent from April to October 1993 were 467.5, 24.7, and 3.3 respectively.

Histological and Stereological Techniques

Biweekly, 20 random individuals from tanks 2 × h were dissected and fixed in Bouin–Hollande's solution (Gabe 1968). After dehydration, the tissue was embedded in paraffin wax (Merck m.p. 56–58 °C). Sections 6-μm thick, separated by at least 100 μm (Heffernan and Walker 1989), were cut and stained in Cleveland–Wolfe dye (Gabe 1968).

Quantitative stereological methods were used to determine size distribution of oocytes for each sampling date. Methods were those described by Morvan and Ansell (1988), Paulet and Boucher (1991), and Pazos et al. (1996). Video print at 200× magnification were obtained from 12 randomly selected fields from each histological section of an ovary. All oocyte profiles were marked with a black felt tip pen. The size of each oocyte was obtained by video camera and processed by Visilog 3.21 image analyzer (Noesis, France). These analog video signals were converted to a binary format using upper and lower gray-level thresholds set by the operator (Heffernan et al. 1989).

To obtain the oocyte size distribution, 90 to 200 complete oocyte profiles of each female were measured, according to Morvan and Ansell (1988) and Laruelle et al. (1994). Statistical errors are minimized by taking sufficient measurements. The image analyzer was used to determine the profile areas and a theoretical diameter (D_t) calculated, based on the assumption that each section is a circle: $D_t = (4s/\pi)^{1/2}$. A frequency distribution of the profile diameters in the 5-μm size class was obtained for each animal. The Saltykov (1958) algorithm, a non-parametrical method applicable to polymodal distributions was used to obtain the real oocyte-size distribution from the profile diameter distribution (Williams 1981, Morvan and Ansell 1988, Paulet and Boucher, 1991).

Oocyte size–frequency data for a number of animals or samples can be considered as a ($r \times c$) contingency table where r is the number of individuals (or samples) and c is the number of size classes (Grant and Tyler 1983). The statistic G was computed. G is distributed similarly to χ^2 with $(r - 1)(c - 1)$ degrees of freedom (v). If a significant value is obtained, it can then be concluded there is heterogeneity within the samples. It is possible to determine which size classes and which individuals (or samples) contribute most to the value of G by examining adjusted residuals in a contingency table (Haberman 1973, Grant and Tyler 1983). A positive residual indicates that the frequency of oocytes in that size class is greater than expected. ANOVA and the Student–Newman–Keuls (SNK) 'a posteriori' procedure were used to test for significant differences between means (Sokal and Rohlf 1981). Normality of variables was determined using the Kolmogorow–Smirnof test (Sokal and Rohlf 1981) and homogeneity of variance with the Bartlett test (Sokal and Rohlf 1981). Statistical analyses were performed using the SPSS statistical package.

RESULTS

Water Quality

Figure 1 shows water temperature and chlorophyll *a* levels. Temperatures ranged from 11 °C to 20.3 °C. Chlorophyll *a* levels varied between 2.8 and 5.0 μg/L. Two phytoplankton blooms were recorded during the experimental period, one in June and another in September. Salinity was stable at 35–36‰.

Survival, Growth Rate, and Condition Index

Mortality was 24% during the acclimation period (mainly in the smaller seed) but less than 18% throughout the course of the experiment. No significant effects of experimental conditions on survival were observed. During the acclimation period the seed underwent rapid growth (Fig. 2A) reaching an average live weight of 360.1 ± 127.2 mg SD ($n = 100$) and length of 14.3 ± 1.7 mm SD in 60 days.

Growth rate of the seed in the effluent water was significantly faster (Table 1, Fig. 2A) and showed better condition indices than clams grown in sea water (Fig. 2B). Duncan's test showed there were significant differences ($P < .05$) in clam growth rate between different flow rates.

Length and weight increases were lowest in the control tanks, ($K = .001$, $n = 90$). In the 1 × h tanks, the final weight and

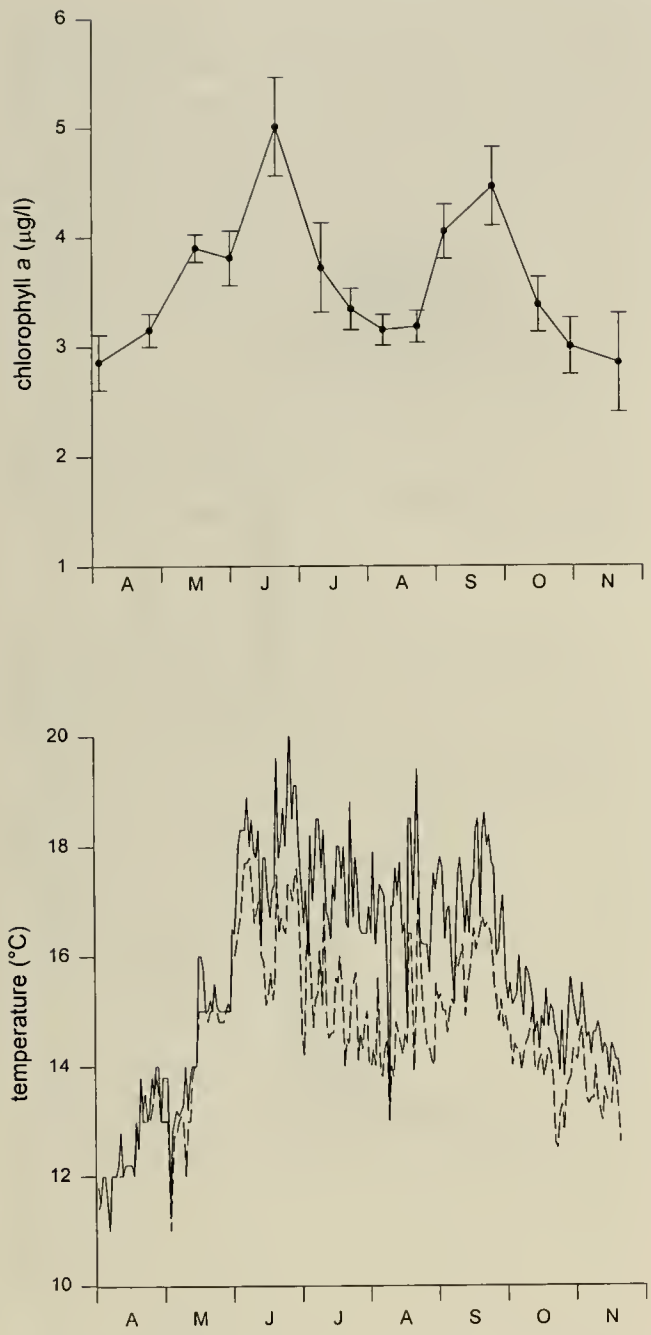

Figure 1. Biweekly mean values for chlorophyll *a* concentrations (n = 3) and maximum and minimum daily temperatures in the wastewater from turbot culture.

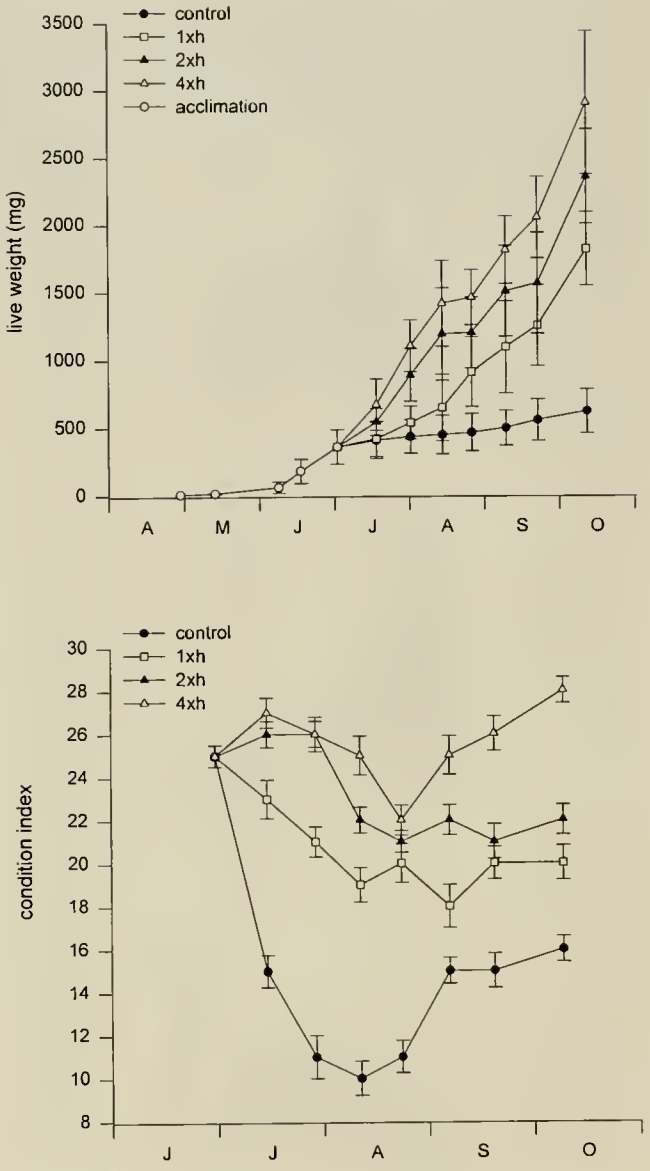

Figure 2. Mean live weight ± SD (A) and condition index ± SD (B) of *Venerupis pullastra* seed (n = 90) reared in turbot culture wastewater and control tanks.

length of the seed (an average of 1,823 ± 273.2 mg SD and 23 ± 1.9 mm SD) (K = .0102, n = 90) were greater than that in control tanks. In the 2 × h tanks, clam weight and length was 2,361 ± 351.4 and 24.8 ± 1.4 mm SD on October 20. Shell length (27.4 ± 1.8 mm SD) and particularly live weight (2,907 ± 530.9 mg SD) of *V. pullastra* was higher in the 4 × h tanks and the fastest growth rate was also obtained in these tanks (K = .0445, n = 90).

CIs in the experimental tanks (1 × h, 2 × h, and 4 × h), ranging from 18 to 28 (Fig. 2B), were significantly higher than in the control tanks where the CI fell drastically in the first two weeks from 25 to 10, reaching the maximum value of 16 in September.

Gonad Development and Stereology

As expected, microscopic examination of histological preparations showed that sex ratios did not diverge significantly from a 1:1 ratio (Pérez–Camacho 1980), except for the August 11 sample in which only 5 females were found. Gonadal sections showed females were in different developmental stages at each sampling and that oocytes were in different stages of maturation in individual females. Males showed a much higher synchronization of development and gametes were in the same phase of development in each male. Gametogenic activity was evident throughout the experimental period and it appears several spawnings occurred during this period.

Figure 3 shows the distribution of oocyte size-frequency during the experimental period. Greatest mean oocyte diameter was found in the August 11 sample, where the 45–50 µm and 50–55 µm classes formed 50% of the total oocytes measured. In the previous

TABLE I.

Growth of *Venerupis pullastra* reared in a turbot culture effluent at different flow rates

Date	DMW ± SD (mg)	Length ± SD (mm)	Height ± SD (mm)
Control			
30 June	36.2 ± 1.8	14.3 ± 1.7	8.4 ± 1.0
14 July	34.4 ± 0.4	15.0 ± 0.9	9.1 ± 1.0
28 July	37.2 ± 1.2	15.4 ± 0.9	9.3 ± 0.9
11 August	39.7 ± 0.6	15.4 ± 1.1	9.4 ± 1.0
25 August	60.9 ± 2.1	15.5 ± 0.9	9.4 ± 1.0
8 September	64.2 ± 0.9	15.9 ± 1.0	9.7 ± 1.0
21 September	69.6 ± 0.6	16.3 ± 1.1	10.0 ± 1.1
20 October	79.9 ± 2.1	17.5 ± 1.3	10.8 ± 1.2
1xh			
30 June	36.2 ± 1.8	14.3 ± 1.7	8.4 ± 1.7
14 July	40.4 ± 2.7	14.6 ± 1.7	8.9 ± 1.0
28 July	41.6 ± 3.8	16.2 ± 1.2	9.8 ± 0.8
11 August	51.5 ± 1.4	16.7 ± 2.2	10.4 ± 1.3
25 August	68.9 ± 2.5	18.9 ± 1.9	11.5 ± 1.1
8 September	76.5 ± 2.0	19.9 ± 2.4	12.1 ± 1.5
21 September	92.4 ± 3.7	20.8 ± 2.3	12.7 ± 1.4
20 October	126.6 ± 2.9	23.0 ± 1.9	14.3 ± 1.2
2xh			
30 June	36.2 ± 1.8	14.3 ± 1.7	8.4 ± 1.0
14 July	46.7 ± 8.3	16.4 ± 1.1	10.0 ± 0.7
28 July	80.8 ± 5.3	18.9 ± 1.1	11.6 ± 0.9
11 August	92.3 ± 15.1	20.6 ± 1.9	12.8 ± 1.2
25 August	96.2 ± 9.7	20.6 ± 1.4	12.6 ± 1.0
8 September	124.8 ± 13.5	22.5 ± 1.5	13.8 ± 1.1
21 September	127.6 ± 8.7	22.6 ± 1.8	13.9 ± 1.2
20 October	180.7 ± 7.9	24.8 ± 1.4	15.3 ± 1.0
4xh			
30 June	36.2 ± 1.8	14.3 ± 1.7	8.4 ± 1.0
14 July	63.2 ± 4.9	17.1 ± 1.5	10.5 ± 1.0
28 July	103.7 ± 11.1	20.1 ± 1.1	12.3 ± 1.0
11 August	115.5 ± 7.9	21.9 ± 1.3	13.4 ± 0.7
25 August	138.8 ± 6.5	22.4 ± 1.0	13.5 ± 0.7
8 September	161.5 ± 10.3	23.7 ± 1.1	14.5 ± 0.7
21 September	168.6 ± 3.5	24.2 ± 1.0	15.1 ± 0.9
20 October	191.1 ± 2.1	27.4 ± 1.8	15.9 ± 1.0

Values are mean ± SD (n = 90).
DMW = Dry meat weight.

sample (July 28), mature eggs were not found, indicating a rapid redevelopment following spawning.

The dynamics of oocyte cohort maturation can be studied from the position of positive residuals in the contingency table (Grant and Tyler 1983). The contingency tabled ($r \times c$) of adjusted residuals for oocyte size-frequency is shown in Table 2, where r was eight samples and c 11 oocyte size classes. Maturation of the gonad involved a displacement of the positive residuals to greater size classes (35–50 μm) and spawning to smaller classes (0–35 μm).

A complete spawning took place between the July 14 and 28 samples, after which the gonad underwent rapid recovery, as shown by the large number of oocytes with diameters between 35–50 μm found in the August 11 sample (Fig. 3, Table 2). This indicates that a second spawning, possibly a partial spawning, was imminent. Although most of the oocytes on August 25 were in the 5–10 μm and 10–15 μm size classes, mature eggs (35–40 μm) were also present in the follicles. Subsequently, a period of gonadal redevelopment occurred, although it was slower than the one that took place between July 14 and 28. A third spawning, although small, occurred between August 25 and September 8 possibly as a result that this of oocytes of the larger size classes in the previous sample that were not released because they were not mature. Between September 8 and 21, another gonadal recovery was observed that was confirmed by the presence of oocytes of the 0–5 μm class and others in the 30–40 μm class. Finally, a fourth spawning occurred between September 21 and October 20. This spawning was larger than the previous one and had positive residual values coinciding with the larger oocyte size classes (Table 2). Data presented suggest the existence of multiple spawning cycles that is characteristic of this molluscan species.

The degree of synchrony between the stage of oocyte maturation in a sample is indicated by the values of the mean oocyte diameter variation coefficient (CV = 100* ($s/\bar{x}$)). In general the higher synchronization occurred in the earlier gonad development stages and the greatest values of the variation coefficient in the spawning periods.

DISCUSSION

Venerupis pullastra acclimated well to effluent water from turbot culture operations and had better growth than those held in the control tanks. An important objective of this study was to determine the effect of effluent flow rate on the growth. Spencer (1988) demonstrated the influence that flow rate has on the growth of juveniles oysters in experimental outdoor pumped upwelling systems. This author reported that the effect of flow rate on growth depends on the food concentration in the water. For commercial scale culture of *V. pullastra*, a balance needs to be maintained between sufficient flow and available food in the effluent. Insufficient water exchange could cause an excess of organic matter that would adversely affect water quality (low oxygen levels, increased ammonium concentration, etc.), which could induce stress on the clams.

Cultivation density is an important parameter to be considered when evaluating growth rate of a population in a particular site (Spencer et al. 1991). Final density attained in the present work was 5.5 kg/m^2 (flow 4 × h), which is greater than that found in extensive culture of this species in the natural environment where 2.5 kg/m^2 is not surpassed (Walne 1976, Cerviño et al. 1993, Robert et al. 1993, Pech et al. 1993). Our results in the 4 × h tanks showed that this stocking density (5.5 kg/m^2) produced growth rates similar to those reported under natural conditions by several authors for the same species (Pérez-Camacho 1980).

Two other important parameters that influence growth and gametogenic development in bivalve molluscs are temperature and available food (Sastry 1979, Bodoy et al. 1980, Maitre-Allain 1982, Beninger and Lucas 1984, Wilson and Simmons 1985, Laing et al. 1987, Ruiz et al. 1992).

Several authors (Mann and Glomb 1978, Mann 1979, Wilbur and Hilbish 1989, Albentosa et al. 1994) have studied the effect of temperature on the growth in bivalves. Albentosa et al. (1994) showed the optimum temperature for growth of *Venerupis pullastra* spat was about 20 °C when sufficient food is available. When the temperature rose to 25 °C, ingestion decreased, ammonia excretion and respiration rate increased, and the smallest individuals were more sensitive to these changes. Shpigel and Fridman (1990) studied growth and gonad cycle in the clam *Ruditapes philippinarum* held in effluent water from an intense fish cultivation in the

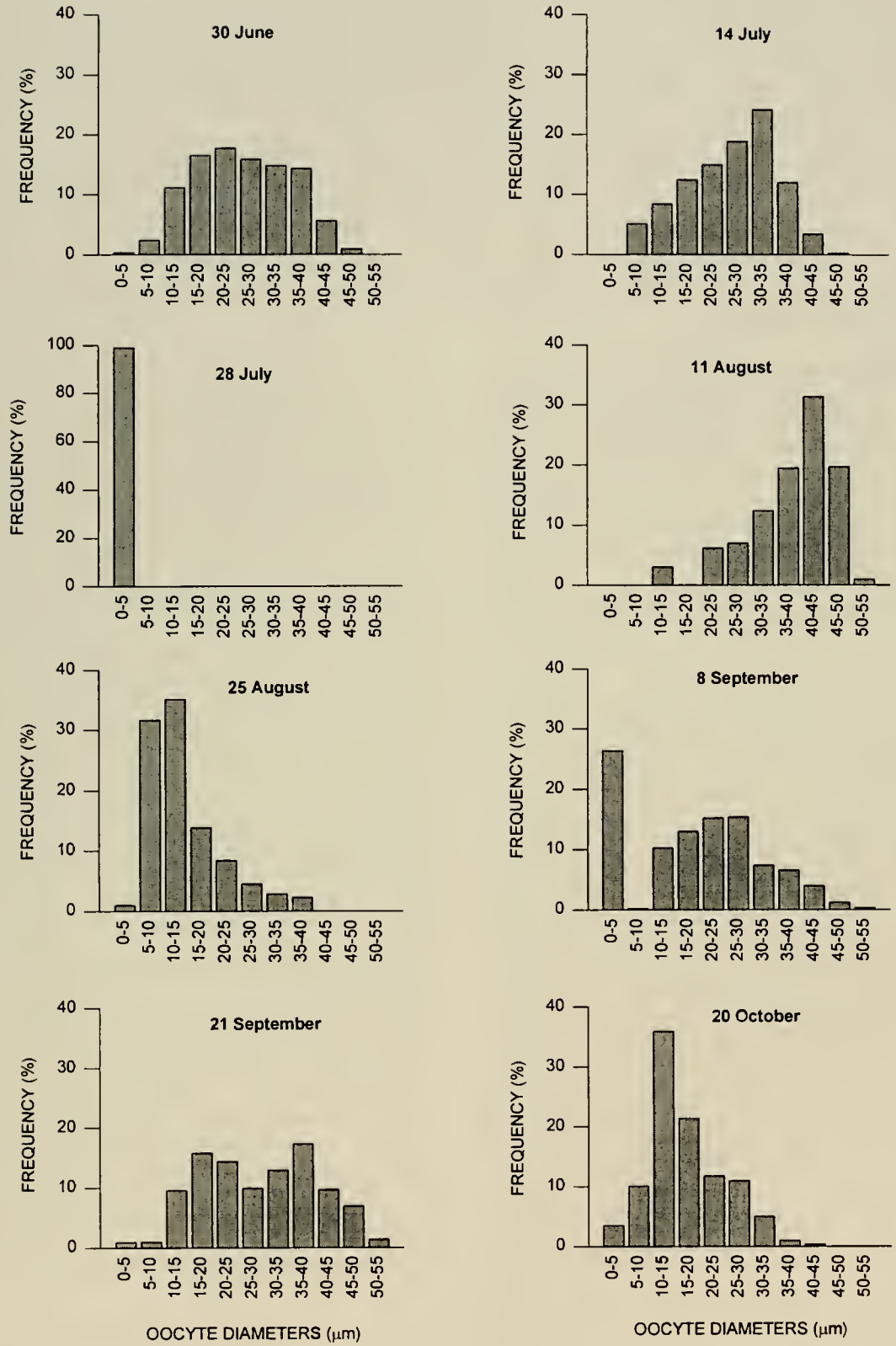

Figure 3. Distribution of oocyte size-frequencies (classified in size ranges of 5 μm) at each sampling of *Venerupis pullastra* seed reared in 2 × h wastewater flow.

TABLE 2.

Contingency table of adjusted residuals for oocyte size–frequency data in *Venerupis pullastra* reared in a turbot culture effluent.

Date	Oocyte diameter size class (μm)									
	0–5	5–10	10–15	15–20	20–25	25–30	30–35	35–40	40–45	45–50	50–55
30 June	−14.54	−7.47	−4.15	**4.32**	**6.33**	**4.69**	**4.43**	**7.65**	**1.39**	−3.10	−2.06
14 July	−22.66	−5.30	−10.78	−0.56	**4.57**	**12.59**	**24.16**	**6.79**	−3.82	−7.56	−2.48
28 July	**80.46**	−8.88	−13.09	−11.96	−11.71	−11.46	−11.06	−9.64	−7.05	−4.63	−1.65
11 August	−10.93	−7.38	−8.71	−9.94	−4.91	−3.97	**0.99**	**9.82**	**31.80**	**31.23**	**3.52**
25 August	−17.52	**44.85**	**27.57**	**1.47**	−5.92	−11.23	−12.92	−11.22	−10.37	−7.29	−2.60
8 September	**21.93**	−15.19	−7.30	**0.26**	**4.74**	**5.92**	−6.75	−4.33	−2.30	−3.67	**0.72**
21 September	−13.98	−9.61	−5.92	**3.44**	**2.38**	−2.37	**2.17**	**11.81**	**8.72**	**12.79**	**8.03**
20 October	−9.72	**3.33**	**20.02**	**8.77**	−0.62	−0.98	−6.75	−9.41	−7.17	−5.11	−1.82

Positive residuals are in bold type.

Gulf of Eilat (Aqaba, Israel) and showed the importance of temperature in evaluating culture under such conditions. They showed that the high temperatures (>27 °C) and salinities of 40 ppt, which occur in the Gulf of Eilat in July and August, produced high mortality rates and a decrease in growth of *Ruditapes philippinarum*. However, the lower temperatures and salinities found in this study are more compatible for culture of this species. As a consequence there was better growth, lower mortality rates, and normal development of the gonadal cycle.

Although oxygen levels were greater in the tanks with higher effluent flows, the injected oxygen in the fish tanks ensured that good levels reached the clams and were not a limiting factor for growth.

Differences in growth may be attributable to differences in available food in the experimental tanks. The high ingestion rate of *Venerupis pullastra* seed (Beiras et al. 1993) might explain this. Poor results found in the August 25 sample for the 2 × h and 4 × h tanks are likely to have been due to the intermittent water supply for 5 days caused by pump failures. This would cause a lower effluent flow and a reduced amount of food. This reduction in growth was not found in the 1 × h tanks where increases in both length and weight were recorded. There are two possible explanations for this difference: smaller size of the individuals in the 1 × h tanks (mean live weight of 911.3 ± 258.0 mg SD and mean length of 18.9 ± 1.8 mm SD) and a 20% mortality that occurred in the smaller animals in these tanks that would produce an increase in mean length and weight. The mortality may have been due to lack of food and more importantly to high levels of metabolites (Albentosa et al. 1994) in these tanks due to lower flow rates.

Results of the analysis of variance for weight, length and height of the seed indicated that significant differences existed ($P < .05$) between tanks 2 × h and 4 × h, but that growth in 4 × h tanks was only 18% greater than the 2 × h tanks although it received twice the amount of effluent.

Results showed that condition index increased with higher flows of effluent. Condition index in animals depends primarily on the amount of food available in the environment (Lucas and Beninger 1985). Condition index of clams in the 1 × h tanks declined at the beginning of the experiment. This may have resulted as a consequence of the acclimation period where the seed was held in a higher water flow (1,120 L/h). Condition indices of clams held in tanks with flows 2 × h and 4 × h were much greater than those of clams in the control tanks and showed a progressive increase in value, except between August 11 and 25 for reasons already given. In general, condition indices of seed held in effluent water were superior to those of seed held in the natural environment. Brown and Hartwick (1988) studied the effect of temperature, salinity and food on *Crassostrea gigas* culture and found that thickness of the shell is directly related to food abundance. When food was scarce, growth and dry weight of the soft parts were low with respect to dry weight of the shell and the internal volume was reduced thus increasing shell thickness.

Many investigators have studied the influence of quantity of available food on gametogenesis in marine bivalves (Sastry 1975, Bodoy et al. 1980, Himmelman 1980, Maitre-Allain 1981, Beninger and Lucas 1984, Wilson and Simmons 1985, Ruiz et al. 1992). Velez and Epifanio (1981) reported that the experimental manipulations of food quantity changed the reproductive cycle of the mussel, *Perna perna*. Newell et al. (1982) studied growth and reproduction of *Mytilus edulis* from different locations with the same temperature characteristics and showed that individuals exhibited different reproductive patterns that were due to differences in trophic levels (measured as chlorophyll *a*), which could greatly delay the reproductive cycle at the different sites.

Pérez-Camacho (1980) showed that *Venerupis pullastra* had a characteristic rapid growth in a relatively short period of time, which probably explains why this species reaches sexual maturity at such an early age compared to other similar species such as *Ruditapes decussatus* (Figueras 1957).

When food is abundant, storage of energy reserves is simultaneous with fast maturation of the gonad, spawning, and a rapid regeneration of the gonad to maturity. In our experimental tanks, there was an abundant and constant supply of food and the seasonal rapid recovery of the gonad found in the August 11 sample after the first spawning occurred between July 14 and 28.

Finally, the gonadal cycle of clams held in the effluent from fish ponds developed without apparent disruption. Total maturation and apparent normal development of gametes was observed; thus a complete life cycle of *Venerupis pullastra* may be attained by holding them in effluent fish ponds. We believe this was possible because of the favourable and stable parameters of the effluent (temperature, salinity, oxygen, chlorophyll *a*, etc.) that occurred throughout the experiment.

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BALANCING TRADEOFFS BETWEEN PREDATOR PROTECTION AND ASSOCIATED GROWTH PENALTIES IN AQUACULTURE OF NORTHERN QUAHOGS, *MERCENARIA MERCENARIA* (LINNAEUS, 1758): A COMPARISON OF TWO COMMON GROW-OUT METHODS

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ABSTRACT Poor survivorship of *Mercenaria mercenaria* seed clams is the chief obstacle hindering economically successful grow-out operations of hard clams. While much effort has been invested in decreasing such mortality, increasing protection of clams often results in a reduction in their growth. We examined how the mortality and growth of seed clams during the first year of culture differed between two common grow-out methods: tented, nylon-mesh bags and mesh-covered bottom areas. We also determined how initial seed size modified these relationships. Specifically, three initial seed sizes (SL = 10.9, 13.0, and 14.8 mm) were grown separately in 1.2 × 1.2 m (9.4-mm) mesh bags tented with a 30-cm-long PVC stake in the center. At the same time, two initial seed sizes (SL = 12.8 and 14.9 mm) were grown in 1.2 × 1.2 m bottom beds covered with 7.0-mm polypropylene mesh. All treatments were stocked with 700 seed clams. Clam survival in tented bags was enhanced by increasing initial planting size. Small seed (10.9 mm) had a mean survival rate of 76.6% and large seed (14.8 mm) had a mean survival rate of 93.1%. Survival was greater in nylon bags than mesh-covered bottom beds when similar initial seed sizes were used. When data for medium (13.0 mm) and large (14.8 mm) initial seed sizes were pooled, the mean survival in tented bags was 90.1%. Survival in the mesh-covered bottom-beds for similar initial clam sizes (12.8 mm and 14.9 mm) was 71.7%. Clam growth (in SL) was 21.7 % greater within mesh-covered bottom beds than in nylon bags. Increasing initial seed size enhanced clam growth slightly in both grow-out methods. This study demonstrates a method to increase survival using tented nylon-bags for the first year of grow-out as compared to mesh-covered bottom-beds; however, there is a growth penalty associated with this increased survival. The project also suggests a minimum initial seed size (>12 mm SL) that should be attained from a nursery system before planting in tented bags or bottom beds because greater survivorship associated with larger initial clam size more than compensated for the increased cost of planting larger seed clams.

KEY WORDS: *Mercenaria mercenaria*, quahog, aquaculture, survivorship, growth penalty, flow, bottom culture

INTRODUCTION

There has been substantial interest and growth in hard clam (*Mercenaria mercenaria*) aquaculture in several states along the Atlantic and Gulf Coasts over the last three decades. Despite increased awareness of the potential of aquaculture and reports of success in clam aquaculture in some states (notably Florida and Virginia), growers in many areas, including North Carolina, have not fully realized the potential of the industry (Diaby 1997). Perhaps the greatest inhibitor to the establishment of an economically viable culture industry is controlling clam mortality caused by predatory crabs (Carriker 1959, Menzel et al. 1976, Whetstone and Eversole 1978, Castagna and Kraeuter 1981, Walker 1984, Gibbons and Castagna 1985, Peterson et al. 1995, Marelli and Arnold 1996, Kraeuter et al. 1998, Smith and Langdon 1998). Although several techniques have been proposed to decrease predation, it still remains a critical factor in determining the success of clam-aquaculture operations (Fernández et al. 1999). While the success of many of these predator-exclusion techniques has varied, the methods that have been successful at increasing clam survivorship are generally associated with reduced clam growth (i.e. a growth penalty). Unfortunately, several studies have failed to examine the relationship between the efficacy of predator-exclusion methods and any associated growth penalty of using such devices (e.g. Castagna and Kraeuter 1977, Gibbons and Castagna 1985, but see Peterson et al. 1995, Kraeuter et al. 1998).

Of the methods proposed to decrease predation on seed clams, three of the most common are the use of mesh netting to cover clam beds (e.g. Manzi et al. 1981, Kemp 1991), the use of gravel or shell hash (e.g. Castagna and Kraeuter 1977, Summerson et al. 1995), and more recently, the use of nylon-mesh bags (Kraeuter et al. 1998, Fernández et al. 1999). Summerson et al. (1995) documented a substantial increase in survivorship with the addition of gravel substrate to clam beds compared to beds without gravel. Yet the percentage of marketable clams after 36 mo was 20% to 25% higher in beds without gravel substrate compared to beds with gravel at the same planting density. The mechanism by which the introduction of gravel reduces growth has yet to be fully explained; however, disruption of the fluid boundary layer (Weissburg and Zimmer-Faust 1993) may be partially responsible. Reduced growth may also be a problem when using mesh netting or cages if fouling algae and animals settle onto the mesh (as is the case in many aquaculture areas). Fouling algae reduce the flow of water and consequently the delivery of food to animals under the mesh (Wildish and Kristmanson 1984, Paul and Davies 1986). Encrusting animals that settle on the mesh may also directly compete with clams for food (Peterson 1979, Fernández et al. 1999). Growth reductions may be further exacerbated by planting seed at densities that are too high because food depletion of bivalves at higher densities has been shown to be problematic (Peterson and Black 1987, Summerson et al. 1995). Planting seed at high densities can also result in reduced survivorship as a consequence of density-dependent predation by crabs (Eggleston et al. 1992). Clams

grown in mesh bags have shown reduced mortality in seed grow-out trials (Kraeuter et al. 1998, Fernández et al. 1999); however, comparisons of survivorship and growth with alternative grow-out methods have yet to be examined.

Other techniques that have received attention include the use of rafts, cages, (Manzi et al. 1981), grow-out trays or racks (Eldridge et al. 1976, Eldridge et al. 1979), and biological controls. Biological controls tested include: (1) the introduction of a predator that consumes crabs, but not clams (Castagna and Kraeuter 1981, Jory et al. 1984, Bisker and Castagna 1989) and (2) increasing the size of seed clams at planting (Kraeuter and Castagna 1985, Peterson 1990, Peterson et al. 1995). In a previous demonstration project, we found that initial seed size appeared to affect the eventual survival (Hooper unpubl. data). These findings are in general agreement with others who have suggested survival differences related to initial seed sizes (Kraeuter and Castagna 1985, Peterson et al. 1995). If initial seed size is directly related to survival, the nursery phase of clam aquaculture could be adjusted to ensure maximum survival.

In this paper we report the results of grow-out experiments that examined the importance of initial seed size and the efficacy of tented, nylon-mesh bags in bottom culture of *M. mercenaria*. The wide use of mesh netting to cover bottom areas warranted the comparison of this "industry standard" with the nylon-bag technique. Specifically, this paper examines whether there are differences in terms of survival, individual growth, or total yield between bottom grow-out of clams using tented, nylon-mesh bags (nylon bags) and traditional mesh-covered bottom areas (bottom beds). Further, we investigate how these relationship change with size of the initial seed clams. We predicted that any increase in survival would outweigh any growth penalty when seed clams were grown in nylon bags as compared to bottom beds.

MATERIALS AND METHODS

Experimental Design

In August 1998, seed clams (4–6 mm) were obtained from Atlantic Farms, Inc., South Carolina and placed into a nursery system on the premises of Hooper Family Seafood, Smyrna, Carteret County, North Carolina. By October 1998 the clam seed had grown sufficiently to be graded into three distinct size classes for our experimental purposes (small, mean SL = 10.9, medium, mean SL = 13.0, and large, mean SL = 14.8 with SL being the maximum measurement along the anterior-posterior axis). Differences in clam seed size at the end of the nursery phase were attributed to slight variations in flow within the raceways during the previous two months.

In October 1998, three sets of 10 nylon bags of mesh size 9.4 mm (stretch) and measuring 1.2 × 1.2 m (4 × 4 ft) were filled with 700 seed clams. Our experimental density was similar to the density recommended in Fernández et al. (1999). Each of the three sets of nylon bags corresponded to one of three size classes of seed clams (small, medium, and large). After filling, each nylon bag was sealed with a cable tie, staked down on each corner, and raised in the center with a 30-cm-long PVC stake which projected 20 cm above the substrate surface. In January of 1999, the center stake of each nylon bag was removed. A random sample of 100 clams was measured for SL from four of the 10 nylon bags in each size treatment (small, medium, and large) at the beginning of the experiment. A one-factor ANOVA confirmed that the initial size classes were significantly different among the three size treatments ($F_{2,9} = 542.5$; $P < 0.0001$).

In October 1998, we also placed seed clams in bottom beds (1.2 × 1.2 m) and covered the beds with 7-mm polypropylene mesh that was held in place with a 1.3-cm rebar frame staked at the corners. For this experiment, we used two size classes of seed clams in separate plots (medium, mean SH = 12.8 mm and large, mean SL = 14.9 mm) and scattered the clams under the mesh at the same densities as in the nylon bags (700 clams). There were five bottom beds of each initial size (10 bottom beds total). Random samples of 100 clams were measured for SL from two of the five bottom beds of each initial size (medium and large). A two-factor ANOVA confirmed that the two size classes differed in initial SL ($F_{1,8} = 359.5$; $P < 0.001$), but within each initial seed size (medium and large), mean SL did not differ between bottom beds and nylon bags ($F_{1,8} = 1.03$; $P = 0.34$). Thus our overall design allowed for comparisons between the two different methods (nylon bags and bottom beds) and two initial seed sizes (medium and large). The clams were planted in shallow water (<1 m MLW) and on a substrate of firm sand. The 30 nylon bags and 10 bottom beds were interdispersed by assigning random positions within a subplot of North Carolina shellfish lease 9102, located close to the premises of Hooper Family Seafood.

In October of 1999, all nylon bags and bottom beds were harvested. Live clams in each treatment were counted and a random sample of 100 clams was measured for SL. Additionally, a volumetric measurement was taken for each treatment. This involved determining the displaced water volume of 50 randomly selected, live clams from each graded size class for all bags in these treatments in order to estimate the entire volume of each replicate.

Statistical Analyses

Data were analyzed using either one- or two-factor ANOVA for clam survivorship and growth. A two-factor ANOVA was conducted to assess whether grow-out technique (bottom beds versus nylon bags), initial seed size (restricted to medium versus large for this analysis), or their interaction affected survivorship. Our experimental design prevented comparison of all three size classes in the two-factor ANOVA because our bottom bed treatment did not include the small seed clam size class. In order to determine if initial seed size affected the survivorship of seed clams among all three size classes (small, medium, and large) within nylon bags, we performed an additional one-factor ANOVA.

Similar analyses (a two-factor ANOVA for the effect of seed size and grow-out technique, as well as a one-factor ANOVA for the effect of seed size within the nylon bags) were also conducted for the following growth parameters: total clam volume, mean individual clam volume, and mean individual shell length. The data sets in the two-factor ANOVA's assessing the effect of grow-out technique and the interaction between grow-out technique and initial size on clam survival and growth were unbalanced ($n = 5$ for bottom beds versus $n = 10$ for nylon bags). To overcome this potential problem with our analysis, we balanced the data sets according to methods given in Underwood (1997). Our conclusion and the ANOVA tables presented in this paper are based on the balanced data sets. Prior to any of these analyses, data were tested for homogeneity of variances using Cochran's test. In no case was transformation necessary and analysis proceeded with the original data. *Post hoc* contrasts were performed on all significant main effects detected by the ANOVAs or within each group if an in-

teraction was significant using Fisher's PLSD test (Day and Quinn 1989).

RESULTS

Survivorship

Survivorship differed between grow-out methods. Survivorship also differed among initial seed sizes within the nylon bag treatment, but only when small seed clams were included in the analysis. For clams grown in nylon bags, a significant trend of increasing survivorship with larger initial size was detected (Fig. 1; one-factor ANOVA $F_{2,27} = 3.50$; $P = 0.044$). *Post hoc* tests demonstrated that survival was significantly higher in bags seeded with large clams compared to bags seeded with small clams ($P = 0.014$ for Fisher's PLSD); survivorship of medium-sized clams in nylon bags seeded did not differ between either the large or small clams ($P > 0.05$ for both comparisons). Comparison of the method of grow-out and initial size (medium and large) demonstrated a significant effect of grow-out method ($P < 0.001$), but no significant effect of seed size ($P = 0.636$) or interaction between seed size and grow-out method ($P = 0.355$; Table 1 = results of two-factor ANOVA). Survivorship was higher in nylon bags (90.1%) than in bottom beds (71.7%; Fig. 2).

Growth

Within the nylon bags initial size of seed clams had no effect on mean final SL ($F_{2,27} = 2.10$; $P = 0.14$) or individual volume ($F_{2,27} = 1.37$; $P = 0.27$), but did have an effect on total volume of all surviving clams ($F_{2,27} = 5.05$; $P = 0.01$). Individual SL of surviving clams ranged from 32.6 to 31.5 mm (mean SL for large = 32.6 mm, medium = 32.3 mm, and small = 31.5 mm).

For the two-factor ANOVA, which tested the effect of both grow-out method and initial clam size, final SL, individual volume, and total volume of clams differed between grow-out methods ($P < 0.05$; Table 2). While individual volume and total volume did not differ with initial seed size ($P > 0.05$), the effect of initial seed size ($P = 0.038$) was significant for final SL. No significant interactions between initial seed-size and grow-out method were detected; however, there was a marginally significant trend ($P = 0.060$) for the interaction when final SL was examined. Individual clams attained greater SL (Fig. 3) and had higher individual volume (Fig. 4) in the bottom beds than in the nylon bags. Total volume of all surviving clams was also higher in bottom beds compared to nylon bags (Fig. 5). Final SL was the only variable that demonstrated a significant effect of initial seed size. Overall, clam SL was significantly higher in bags (mean 32.6 mm) or beds (mean 40.9 mm) seeded with larger seed clams compared to bags (mean 32.3 mm) or beds (mean 38.1 mm) seeded with medium seed clams (Fig 3). This pattern appeared more pronounced in the bottom bed treatment than in the nylon-bag treatment; the P value for the interaction was marginally significant at the $P = 0.05$ level.

TABLE 1.

Results of the two-factor ANOVA testing whether clam survivorship varied in response to grow-out method, initial seed size (SL), or their interaction.

Factor	df	SS	F	P
Bed vs. tent	1	1541.70	11.73	0.0086
Seed size	1	69.74	0.44	0.5670
Bed vs. tent × seed size	1	137.90	0.87	0.4668
Residual	16	2275.95		

DISCUSSION

Our study compared two of the most common grow-out methods in aquaculture of hard clams: tented, nylon-mesh bags, used primarily along the lower Atlantic Coast, and mesh-covered bottom beds, common in Mid-Atlantic States. While survivorship was considerably higher for clams grown in the nylon bags (+18.4 %) compared to survivorship of clams grown in bottom beds, our overall survivorship in the first year of grow-out was still high with either method (>70%). This high survivorship was most likely a function of the large initial size of our seed clams (>10.9 mm). Survivorship increased with further increases in seed size of clams. While survivorship of large (14.8–14.9 mm) and medium-sized clams (12.8–13.0 mm) did not differ between grow-out methods, small seed clams (10.9 mm) had significantly higher mortality than large seed clams. Similar relationships with initial size of seed

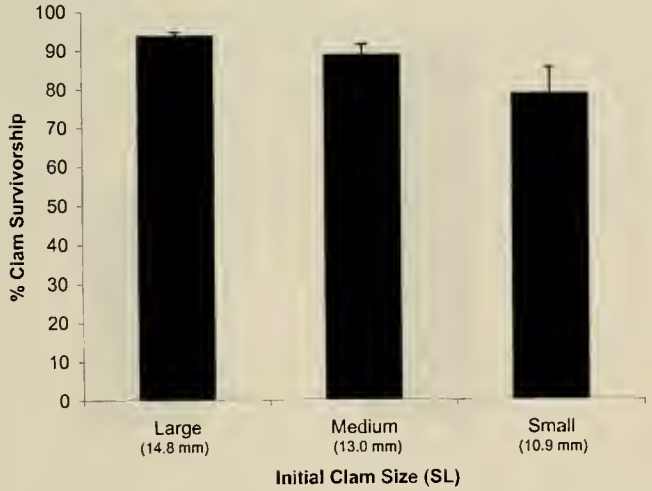

Figure. 1. Percent clam survival in nylon bags after 1 y of grow-out: large, 93.0%; medium, 87.2%; and small, 76.6%. Error bars denote +1 SE ($n = 10$ for each seed size).

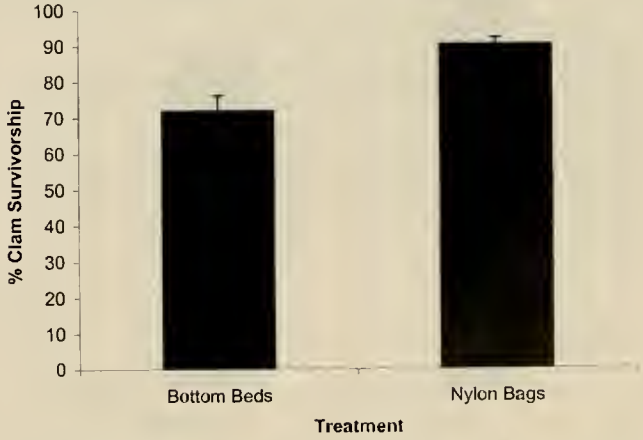

Figure. 2. Percent clam survival in bottom beds (71.7%) versus nylon bags (90.1%) after 1 y of grow-out. Error bars denote +1 SE ($n = 10$ for clams grown in bottom beds and n = 20 for clams grown in nylon bags).

TABLE 2.

Results of the two-way ANOVAs testing whether final individual clam SL, individual volume, and total volume varies in response to grow out method, initial seed size (SL), or their interaction.

	Final Individual Clam SL				Individual Volume			Total Volume		
	df	SS	F	P	SS	F	P	SS	F	P
Bed vs. tent	1	252.49	126.53	<.0001	104.55	60.74	<.0001	8861290.72	8.85	0.0127
Seed size	1	10.66	5.37	0.0376	3.82	2.16	0.1718	1742160.53	1.56	0.2859
Bed vs. tent × seed size	1	8.61	4.34	0.0596	3.33	1.96	0.2065	321309.25	0.37	0.6586
Residual	16	33.37			27.79			16543835.07		

clams and survivorship have been shown by others (Walker 1984, Kraeuter and Castagna 1985, Peterson et al. 1995, Marelli and Arnold 1996). The increase in profit as a result of higher survivorship of large seed clams more than compensates for the additional cost of achieving larger clams in raceways before planting (assuming 95% survivorship after year 1 for all clam sizes). Therefore, evidence from our study indicates that the optimal size for planting in North Carolina is between 12.8 and 14.8 mm (SL). In order to compare our findings with other studies which report clam planting sizes in SH (with SH being the maximum measurement from the umbo to the ventral margin), we estimated the ratio of SH/SL at .875 from empirical data and converted our optimal range to SH (~11.2–13.0 mm). This approximate size range is smaller than the 15- to 20-mm (SH) range reported by Menzel et al. (1976), Whetstone and Eversole (1978), Eldridge et al. (1979), and Walker (1984), but substantially larger than the 8-mm (SH) size that Marelli and Arnold (1996) reported as the size where clam predation becomes insignificant in Florida's Indian River lagoon. Optimal planting size is influenced by factors such as the local predator community and growth rates (which will vary with food availability and food delivery rates), limiting the appropriateness of one particular seed size for multiple regions. Further research on the influence of these factors on seed survival coupled with a greater understanding of the local dynamics of a system should enhance predictive models about the profitability of clam aquaculture ventures for any specific region.

Differences in growth between the two grow-out techniques were opposite of the survivorship results (higher growth occurred in bottom beds, which had lower survivorship), a pattern that has been reported in other studies (e.g. Summerson et al. 1995). Overall, growth was 21.7% higher in bottom beds than in the nylon bags. After 1 y, large seed clams had a final SL of 41 ± 2 mm and medium size clams had a final SL of 38 ± 2 mm in bottom beds, whereas in the tented bags, large seed clams had a final SL of 33 ± 1 mm and medium-sized seed clams had a final SL of 32 ± 1 mm. Final differences between large and medium-sized seed clams in bottom beds reflected the initial size differential of the two groups (~2.1 mm). The initial difference between size in seed clams grown in tented bags was virtually absent at the end of 1 y of growth. These results suggest that growth rate for large and medium-sized seed clams was reduced in comparison to small seed size, a pattern that was not evident in the bottom beds.

Given that the patterns in growth and survivorship were opposite, the question of whether differences in growth can be explained by density dependent factors merits attention. Fernández et al. (1999) examined the effect of density on growth, measured in terms of shell length, for clams grown in bags similar to the nylon bags we used in this experiment. Their results showed no effect of density when seed clams were placed in bags at densities between

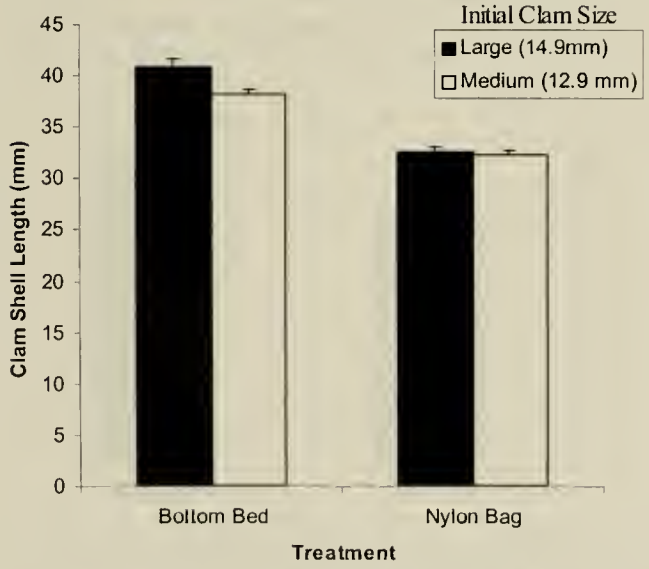

Figure. 3. Final clam shell length (millimeters) for large and medium seed clams in bottom beds and in nylon bags after 1 y of grow-out. Error bars denote +1 SE (n = 10 for each size class within nylon bags and 5 for each one within bottom beds).

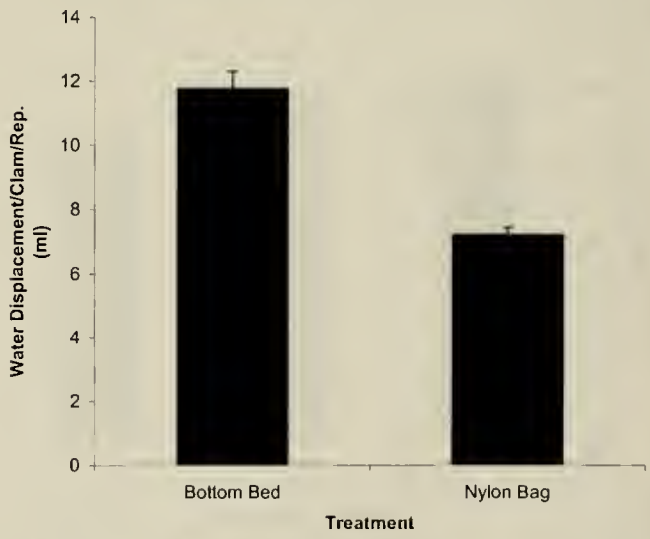

Figure. 4. Individual clam volume after 1 y of grow-out in bottom bed clams (11.74 mL/surviving clam) and tented bag clams (7.194 mL/surviving clam). Error bars denote +1 SE (n = 10 for clams grown in bottom beds and n = 20 for clams grown in nylon bags).

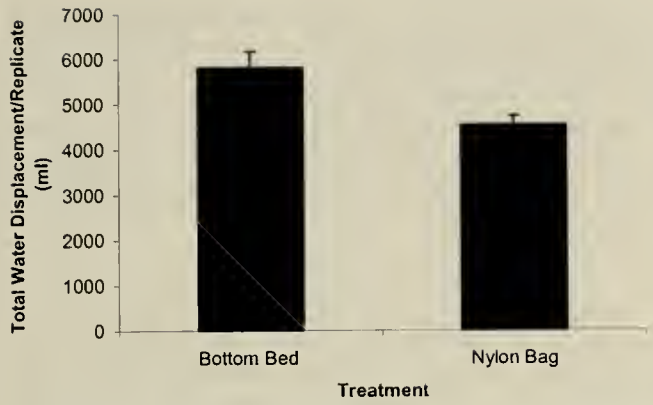

Figure. 5. The mean volume of clams after 1 y of grow-out in bottom beds (5810.7 mL) and in tented bags (4550.1 mL). Error bars denote +1 SE (n = 10 for clams grown in bottom beds and n = 20 for clams grown in nylon bags).

750 and 1,250 clams/bag, densities higher than those used in our study. Further support for our conclusion that the differences in growth between grow-out methods were not derived from differences in density is provided by the examination of our volume data. Individual volume of clams (total volume divided by the number of clams surviving) also showed a significant effect of grow-out method with individual volume higher in bottom beds than in bags. Total volume of all clams within each bag or bed, a parameter that incorporates both survivorship and growth, also differed between grow-out method. If growth were reduced because of density-dependent resource depletion, we would expect both methods to yield similar total volumes of clams (i.e. if resource depletion was a factor, decreases in the number of clams should allow surviving clams to grow faster); however, total volume was greater in the bottom bed grow-out.

Differences in clam growth between the two methods were probably a function of variation in water flow patterns resulting from differences in the physical structure of the methods. Changes in water flow alter the flux of food particles (the product of horizontal advection and concentration of food) over an area usually resulting in differential growth patterns (Wildish and Kristmanson 1997). Differences in mesh size could potentially affect the supply of food; however, our results were opposite what would be predicted under this scenario: mesh size was larger for bags (9.4-mm opening) than bottom mesh (7.0-mm opening). The profile caused by the tenting of the bags would create a strong reduction in flow within the mesh area. Compared to the bottom beds, which would have fairly uniform, more laminar flows, the tented bag would create an area of decreased flow under the bag canopy. Such a difference in flow regime could result in differences in growth between the two methods. Although tenting the bags may drive the pattern of growth differences, tenting was shown to substantially decrease predation in previous trials (Hooper unpubl. data). In these trials, tenting the bags seemed to reduce crab access to clams under the canopy. The survivorship that we report with tenting (77%–93%) is slightly higher than that reported by Fernández et al. (1999) (75%–87%).

A comparison of the economic feasibility of these two grow-out methods for clam aquaculture should consider differences in cost between grow-out methods in addition to the economic implications of the tradeoff between clam growth and survival. The material and labor costs of growing clams in bags are approximately \$0.014 per clam higher than the cost per clam in beds. Therefore, bags must increase clam survivorship by 11.6% in nylon bags to compensate for the higher cost of growing clams in this method (assuming a 5% discount rate, clam price of \$0.15 per clam, 95% survivorship after first year of grow-out, and 3-year grow-out phase). If increased clam growth in bottom beds during the first year of grow-out results in clams achieving legal size more rapidly (i.e. greater percentage of legal clams after 2 and 3 y), nylon bags must increase clam survivorship more than 11.6% to compensate for the economic consequences of the growth penalty during the first year of grow-out. In our study, projections of expected profits suggest that the nylon bag method is more profitable by \$0.016 per planted clam than growing clams in bottom beds even after adjusting the expected percentage of legal clams in subsequent years to account for the growth penalty exhibited within nylon bags.

Certain environmental and economic factors should also influence a grower's decision when selecting a grow-out method: when growing conditions favor high clam growth rates and/or predation intensity is very high, enhancing survivorship could increase a grower's return on their investments. On the other hand, when clams are marketed by weight rather than by count, when risk of clam loss to theft or from hurricane (a particular concern for south Atlantic states) damage is high, and/or when interest rates are high, using a method capable of growing clams more rapidly could increase a grower's profit margin. Studies determining whether growing larger clams in the first year of grow-out necessarily results in achieving a higher proportion of legal clams after 2 and 3 y of culture are needed to more accurately assess the economic consequences of this tradeoff between growth and survival exhibited by the two methods.

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THE REPRODUCTION CYCLE OF *TRIDACNA SQUAMOSA* AND *TRIDACNA MAXIMA* IN RENGIS ISLAND (TIOMAN ISLAND), MALAYSIA

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ABSTRACT Hypodermic extraction of gonadal materials was taken from *Tridacna squamosa* and *Tridacna maxima* populations on Rengis Island (south east of Malaysia in the South China Sea) at approximately monthly intervals between March 1996 and April 1997 to investigate the seasonality of reproduction. A spawning season extending over several months was indicated for both species. The influence of environmental factors such as temperature and salinity on the reproductive cycle of these two species are discussed.

KEY WORDS: Reproductive cycle, *Tridacna squamosa*, *Tridacna maxima*

INTRODUCTION

The populations of giant clams (Family: Tridacnidae) in the Indo-West Pacific is declining due to the combined effects of pollution, environmental degradation, as well as harvesting for commercial and subsistence purposes (Munro 1983). The distribution and abundance of giant clams in Malaysia have been surveyed and documented in Redang Island, Terengganu (Mohamed-Pauzi et al. 1994), Pemanggil Island, Johore (Zulfigar and Tan 1995, Zulfigar and Tan 1996a, Zulfigar and Tan 1996b), and Tioman Island, Pahang (Tan et al. 1998). Low densities of giant clams are found in Malaysia coastal waters and the stocks are gradually decreasing (Zulfigar and Tan 1995). An estimate of 2 clams/100 m^2 *T. squamosa* was found in Redang Island and Kapas Island and 8 clams/100 m^2 *T. squamosa* was found in Perhentian Island (Mohamed-Pauzi et al. 1994). Tan et al. (1998) have reported an average of 6 clams/100 m^2 *T. squamosa*, 13 clams/100 m^2 *T. maxima*, and 21 clams/100 m^2 *T. crocea* in Tioman Island. Only three species of giant clams were reported on Tioman Island, which are *T. squamosa*, *T. maxima*, and *T. crocea*, whereas *Hippopus hippopus* species can only be found on the Johore Islands located south of Tioman Island. *T. squamosa* and *T. maxima* are considered endangered while stocks of *T. crocea* are more abundant. In light of this, restocking of *T. squamosa* and *T. maxima* is needed. Detailed knowledge of the gametogenic cycle will provide necessary data for the successful production of seeds via hatchery techniques. Reproduction studies involving examination of gonad samples from natural populations of giant clams are very limited in Malaysia. The objective of this study is to characterize the reproductive cycles of *T. squamosa* (Lamarck 1819) and *T. maxima* (Roding 1798) at Rengis Island.

MATERIALS AND METHODS

Rengis Island is a small island to the west of Tioman Island, in the southeastern part of Peninsular Malaysia (Fig. 1). Tioman Island has been gazette as a Marine Park by the Federal Government of Malaysia since 1984. Very few scientific or management studies have been carried out on the island. Rengis Island is one of the very few islands on the Peninsular Malaysia with relatively good stock of giant clams (Tan et al. 1988). The island is surrounded by fringing reef, which provide a suitable habitat for the giant clams.

Giant clams are functional hermaphrodites (Wada 1952), first reaching sexual maturity as males, then later developing ovaries which produce eggs and function simultaneously with the testes.

The study on the reproduction cycle of *T. squamosa* and *T. maxima* was conducted from March 1996 to July 1997. Sampling was done using SCUBA. The gonad biopsy technique described by Braley (1984; Fig. 2) was used in this study. A 20-cm hypodermic needle with a plastic plunger was used to extract approximately 1 to 2 g of gonadal tissue from the clams. The needle was inserted vertically into the gonad through the mantle, entering the gonad several centimeters anterior to the exhalent siphon to one side of the medial axis of the clam. The first 30 animals encountered from each species were sampled each month. The sizes of the clams sampled ranged from 35 to 65 cm in shell length

The extracted gonadal material was examined immediately using a light microscope. Egg size was determined under a compound microscope, equipped with a 50 × 2-micron graticule. Gonadal stages for eggs were classified based on a modification of the scheme described by Braley (1988; Table 1).

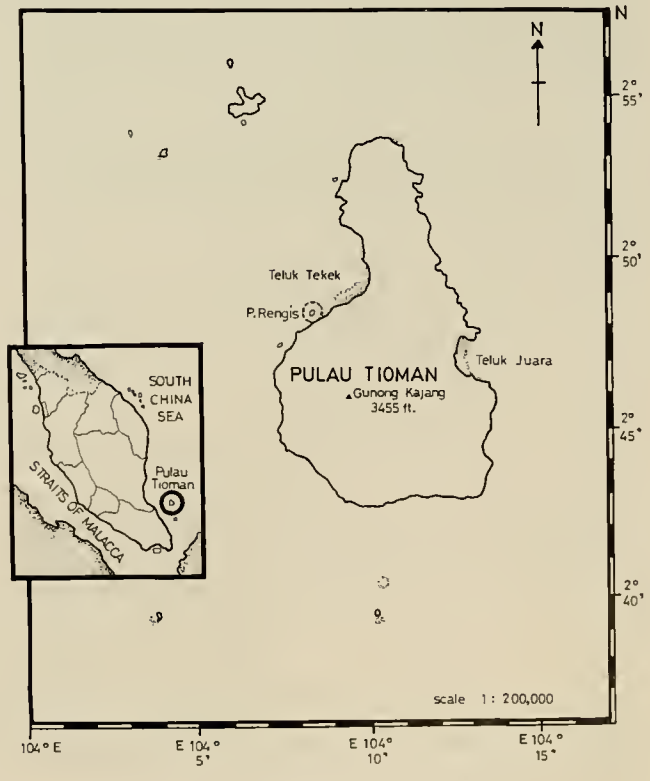

Figure 1. The study location at Rengis Island.

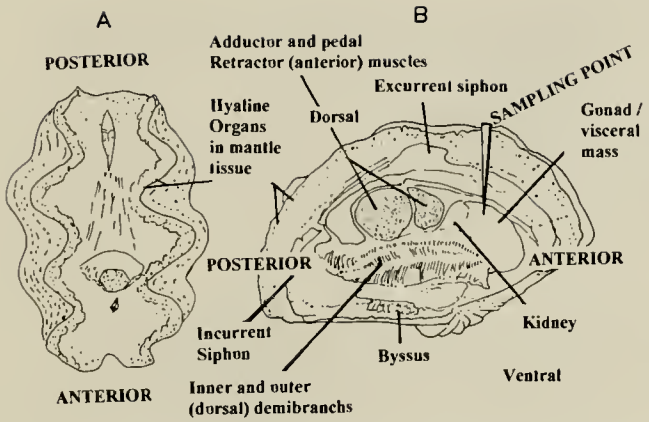

Figure 2. Tridacnid clam, dorsal view (A) and side view (with right valve removed, B). The arrow shows the area from which biopsies of gonadal tissue were taken (modified from Braley 1988).

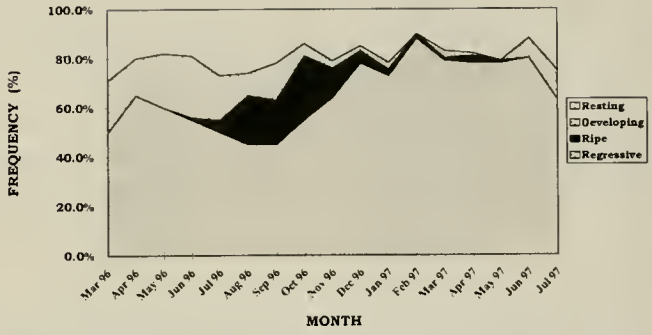

Figure 3. Stages of egg development for *T. squamosa*over a 17-mo period on Rengis Island.

RESULTS

The results indicated that the regressive stage was predominant most of the year for both *T. squamosa* and *T. maxima*. The hypodermic extraction technique tended to extract regressed gametes from within follicles in the gonad, thus biasing the outcome towards the regressive stage (Braley 1988). However, other clear examples of developing, ripe, and resting stages were found in individual clams, indicating that the hypodermic extraction technique does give a true picture of the state of the gonad.

Tridacna squamosa

Figure 3 shows the stages of egg development for *T. squamosa* over a 17-mo period. It is notable that the regressive stage was predominant throughout the study period, including August through November, when ripe gonads were at the peak. The percentage of ripe animals recorded is low throughout the study period, with the highest being 20%. Developing and partially developed biopsy samples were present throughout the year. The number of clams with developing eggs was the highest in June 1996, just before the ripe gonads were observed in August 1996. Clams with resting eggs were present at all sampling occasions.

Tridacna maxima

Figure 4 summarizes the stages of egg development for *T. maxima*; the results are similar to *T. squamosa*. The clams with regressive eggs predominate throughout the study period, followed by the clams with resting eggs. Ripe biopsy samples were recorded throughout most of the study period. A significant proportion of clams with ripe gonads was observed from April to July 1996 and from April to June 1997. The highest percentage of the clams with ripe gonads was 36% in May 1996, followed by 25% in May 1997. The percentage of clams with developing gonads was the highest in April 1996 and April 1997, just before the appearance of a high percentage of ripe gonads in May 1996 and 1997. The clams with resting eggs were present at all sampling periods except in June 1996.

Temperature and Salinity

The water temperature was very stable, and ranged from 29 °C to 33 °C all year round with daily fluctuations to less than 3 °C (Fig. 5). The temperature increased from 31 °C to 32 °C in early March to 33 °C in April. Maximum temperature variation occurred in the beginning of the dry season during the months of April to June due to precipitation and evaporation, as well as minimum turbulence and mixing. The temperature was slightly lower and fluctuated between 29 °C and 31 °C during the wet season from late September to March.

The salinity at Rengis Island at a 5-m depth was also very stable and ranged from 33 ppt (wet season) to 34 ppt (dry season) throughout the study period (Fig. 5). There was no significant variation in salinity at Rengis Island because the island is unaffected by runoffs from large rivers along the peninsular coast.

DISCUSSION

Our study indicates that both *T. squamosa* and *T. maxima* exhibit reproduction periodicity on Rengis Island. However, the period reflecting the highest percentage of clams with ripe gonads

TABLE 1.

Characteristics of eggs at different stages of maturity after Braley (1988).

Stage	Description
Developing	Egg diameter up to 110 μm; generally various sizes in a progressive state; some developing eggs have a distinct peduncle, which is the area of attachment to the follicle wall; and the chorion layer is intact
Ripe	Egg diameter 110 μm or larger; vitellogenesis complete (ova cytoplasm filled with yolk), easily ruptured; ova have intact chorion layers, occasionally ruptured out of the chorion; and large numbers of ripe ova somewhat polygonal in shape due to the dense packing inside ovary follicles
Regressive (post spawning)	Eggs of various sizes, but degenerative; chorion layers often damaged or sometimes not present; and phagocytic amoebocytes present in moderate to large numbers resorbing residual eggs
Resting	Lack of any recognisable eggs; and residual gonadal material and phagocytic amoebocytes were normally present

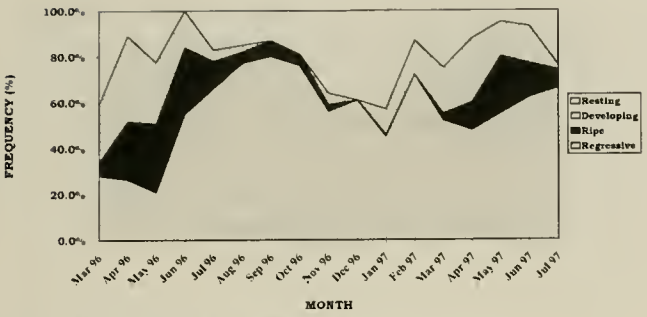

Figure 4. Stages of egg development for *T. maxima* over a 17-mo period on Rengis Island.

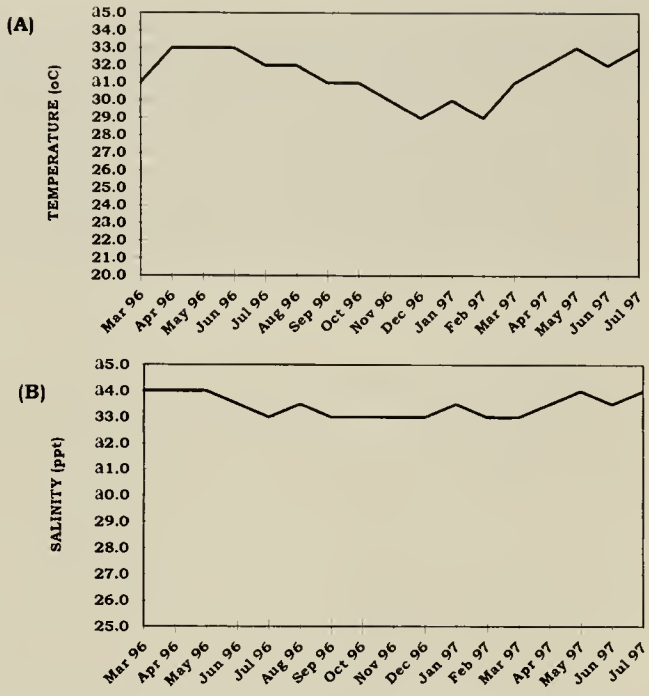

Figure 5. Monthly temperature (A) and salinity (B) changes on Rengis Island in the South China Sea from March 1996 to July 1997.

was different between the two species. The clams with ripe gonads for *T. squamosa* were present from August to November 1996, and for *T. maxima* it was from April to July 1996 and April to June 1977. The differences in the spawning season between the two species may be related to their spatial distributions on the reef. *T. maxima* usually occurs at water depth of 0.5 m (below Chart Datum) to 1.5 m, whereas *T. squamosa* usually occurs at depth of 2.5 to 6.0 m (Tan et al. 1998).

A comparison of the reproduction cycle of both species of tridacnids showed that seasonality is more prominent in *T. maxima* with spawning peak in April to June for both 1996 and 1997.

The season with a high percentage of ripe gonads for *T. squamosa* recorded in this study (August to November 1996) is similar to the spawning period of *T. squamosa* kept in the hatchery in Prachuab Khiri Khan, Thailand (Nugranad et al. 1997). The results obtained in this study are also similar to the reproduction study of *T. squamosa* in Redang Island, Malaysia (also located on the east coast of Peninsular Malaysia), where the highest percentage of clams with ripe gonads was recorded in July and August (Adib et al. 1993). Another study in Republic of Belau, Western Caroline Islands found that the peak months of *T. squamosa* with ripe eggs were in January through March and August through December (Fitt and Trench 1981).

At Rengis Island, the temperature and salinity were very stable. *T. squamosa* spawned at the beginning of the wet season when the temperature declined from 33 °C to 32 °C and at reduced salinity from 34 to 33 ppt. *T. maxima* spawned at the beginning of the dry season when temperature and salinity were slightly elevated. However, the changes in temperature and salinity were not significant and do not seem to have played an important role in the reproduction cycle of giant clams on Rengis Island. Specific environmental cues may be required before the release of eggs occurs (Braley 1988). Cues such as high phytoplankton blooms or rise in temperature may occur in certain years only, resulting in sporadic spawning. Clams that do not release their eggs during the reproductive season could hold the eggs in various states of regression for several months. In the tropical oyster, *Saccostrea cucullata*, the residual gonadal material may be reabsorbed (Braley 1982). Further studies are necessary to determine the specific environmental cues for the giant clams here.

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THE NEW LESSEPSIAN ENTRY *BRACHIDONTES PHARAONIS* (FISCHER P., 1870) (BIVALVIA, MYTILIDAE) IN THE WESTERN MEDITERRANEAN: A PHYSIOLOGICAL ANALYSIS UNDER VARYING NATURAL CONDITIONS

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ABSTRACT The feeding behavior of *Brachidontes pharaonis* (Mollusca, Bivalvia), a new Lessepsian entry in the western Mediterranean, living in a cooling vat of a saltworks system in western Sicily, was assessed by estimating its physiological rates throughout a 6 month-long study (May 1998 to March 1999). Clearance, filtration, ingestion, and food absorption rates were estimated using the biodeposition method and the results correlated to variations in temperature, salinity, and quality and quantity of available food. Measured seston concentrations were on average 81.5 ± 95.5 mg L^{-1}, its labile fraction (estimated as the sum of particulate lipids, carbohydrates and proteins) was on average 0.55 ± 0.07 μg L^{-1}, representing only 15% of the total organics. Phytoplankton biomass, as suspended chlorophyll-a, was on average 0.88 ± 0.4 μg L^{-1}. Mean weight standardised rates of *Brachidontes pharaonis* were clearance rate 1.64 ± 0.82 l h^{-1}, filtration rate 110 ± 107 mg h^{-1} of total suspended material, and egestion rate 0.60 ± 0.16 mg material h^{-1}. There was a mean selection efficiency of 0.50 ± 0.22. Ingested organic matter varied between about 2 mg h^{-1} and 270 mg h^{-1}, and food absorption efficiency ranged between 0.1 and 0.99. The *Brachidontes* feeding process seems to be regulated at the initial filtration stage, and most of the control determines the quantity of absorbed ration. Clearance rate is maintained independent of changes in water temperature and salinity but reflects fluctuations in the quantity and quality of available food. Varying the rate of pseudofaeces production regulated ingestion rate, although this mechanism be fairly inefficient as a response to local environmental conditions. Absorption efficiencies may be sensitive to the balance of biochemical components. The physiological plasticity of *B. pharaonis* as expressed in this study is believed to have played a major role in its ability to reach the western Mediterranean.

KEY WORDS: Lessepsian, *Brachidontes pharaonis*, bivalve mollusc, feeding behaviour, shallow environment, Mediterranean sea

INTRODUCTION

The common Indo-Pacific mussel *Brachidontes pharaonis* (Fischer P., 1870: = *Brachidontes variabilis* Krauss 1848) recently has appeared in the western Mediterranean, reaching the Sicilian coasts through the Suez Canal (Safriel et al. 1980). This hyperhaline mussel is a Lessepsian "new entry" (Por 1971) which, with a few other species [e.g., *Portunus pelagicus* (Linnè 1758) and *Cerithium scabridum* (Philippi 1848)], has been able to cross the Red Sea Barrier (Safriel and Ritte 1977) and reach the temperature eastern Mediterranean.

B. pharaonis was never found in the Mediterranean before the opening of the Suez Canal (1869), and its penetration and consequent diffusion has been rapid, due possibly to human phoresys. The species was first recorded in the Eastern Mediterranean 7 years after the opening of the Suez Canal (Fuchs 1878), and approximately 60 years passed before *B. pharaonis* was recorded along the coasts of Lebanon (Safriel et al. 1980). *B. pharaonis* has been able to cross the barrier of the Levantine Basin and has successfully colonized the western Mediterranean basin. It has now colonized several parts of western Mediterranean coasts (Di Geronimo 1971, Gianguzza et al. 1997) and is becoming a potential resource and space competitor (Safriel and Sasson-Frostig 1988) with its Mediterranean ecological equivalent *Mytilaster minimus* (Poli). *B. pharaonis* has been documented in cooling vats of a saltworks system in western Sicily (Gianguzza et al. 1997, Vitturi et al. in press), where it has extensively colonized hard substrates.

Nevertheless, little is known about small mytilids (*Brachidontes* or *Mytilaster*), which have a low importance for aquaculture (as inedible species) but which represent key species in the recycling of matter and energy in some aquatic ecosystems (Riisgård 1988, Dame 1996).

To our knowledge, there are no previous studies on trophic preferences, adaptability, and physiological plasticity in these bivalve species. Very few data exist on the *Brachidontes* genus (Stern and Achituv 1978), except for some information on *B. exustus* (Riisgård 1988), and there are no data on the feeding activity and physiological features of *Brachidontes pharaonis*. Morton et al. (1988) reported only that *B. pharaonis*, like many other bivalve molluscs, is an active suspension feeder that filters and sorts particles (seston) from the seawater.

Analysis of physiological features is considered among the best tools for investigating plasticity in mollusc bivalves (*sensu* Bayne 1998), especially when experiments are carried out *in situ* with natural food concentrations. The main aims of the present article are thus to describe for the first time some features of the feeding behaviour of *B. pharaonis* estimated using the biodeposition method (Iglesias et al. 1992 and following related literature) and to measure food consumption, rejection, ingestion, and absorption rates throughout an annual cycle in the field. Consequently, the physiological responses of this species are determined under a wide range of ambient conditions of temperature, salinity, and food supply.

Description of the Study Area

This study was carried out between May 1998 and March 1999 in a cooling vat (60,000 m^2; 48,000 m^3; 1 m average depth) of a saltworks system adjacent to the Stagnone di Marsala shallow sound in western Sicily (37°52′ NORD; 12°28′ EAST). *B. pharaonis* has extensively colonized the submerged hard substrates on the saltworks bottom. The soft substrates in the saltworks mainly have been colonized by *Cymodocea nodosa*, whereas the hard substrates are covered with a number of species of macroalgae such as *Laurencia papillosa*, *Padina pavonica*, and *Acetabularia acetabulum*.

MATERIALS AND METHODS

Experimental Design

On the day before the filtration experiments, specimens were collected randomly from the saltworks. Individuals from a 30 ± 1.5-mm size class were selected, cleaned of epibionts and byssus, and then placed overnight in small plastic net-bags in the saltworks water to permit re-acclimatization after the manipulation. In the early morning of the following day, 14 organisms were carefully collected from the acclimatized groups and put into 14 small experimental rectangular chambers (20 × 5 × 5 cm; available volume about 500 mL) with a V-shape bottom. After the valves of the mussels had opened and as soon as their filtration activity was stabilized, the experiments were started. Two chambers without mussels were used as controls. Filtration measurements were carried out in continuous flow, with water from the saltworks continuously pumped into a header-tank (about 50 L). The saltworks water was agitated well by a magnetic stirrer in the tank to avoid particle settling. The continuous flow was regulated at about 180 mL h^{-1} (±20 mL), and this flow was maintained by means of a peristaltic pump placed between the header-tank and the experimental chambers. At this flow rate, little or no sedimentation occurred. Any sedimentation of suspended particles was accounted for by the control chamber (with no animals). After approximately 1 h of active filtration under continuous flow conditions, water samples were collected at the end of the experimental chambers. On completion of the clearance experiments, the organisms were kept for a further 2–3 h in their respective chambers. Separately, faeces and pseudofaeces were carefully collected. This material was filtered onto pre-weighed and ashed GF/F filters, washed with ammonium formate to remove seawater salts, and processed, as described later, to determine the total mass and organic content of the biodeposits.

These experimental procedures allowed clearance rates to be calculated, by applying the following formula:

$CR = f^* [(Ci - Co)/Ci]$ (Labarta et al. 1997, Widdows and Staff 1997)

where CR = the clearance rate in liters h^{-1}

f = the flow rate of water through the chambers (l h^{-1})

Ci = the concentration of labile particulate organic matter (LPOM; mg L^{-1}) in the in-flowing water (determined from the mean of the concentration of LPOM in the water in the tank and in the control chamber)

Co = the concentration of LPOM (mg L^{-1}) in the out-flowing water from the experimental chamber.

The labile fraction of particulate organic matter (LPOM, mg L^{-1}) was calculated from the sum of particulate lipids, proteins and carbohydrates (Widdows et al. 1979, Fichez 1991, Navarro et al. 1993, Sarà et al. 1998).

In order to confirm the estimates obtained using this method, clearance rates were also estimated using the biodeposition method (Iglesias et al. 1992) as follows: CR = (mg inorganic matter produced both as true faeces and pseudofaeces per hour) ÷ (mg inorganic matter per liter in saltworks water). All clearance rates were corrected to a 1 g dry weight standard-sized individual using the weight exponent b (=0.53) reported in Labarta et al. (1997). Results obtained using both methods described were compared and averaged. Production rates of pseudofaeces (namely rejection rates; RRs, mg h^{-1}) were calculated both for inorganic (IRR, mg h^{-1}) and organic (ORR, mg h^{-1}) suspended matter. Also, production rates of true faeces (ER, mg h^{-1}) were calculated for both inorganic (IER, mg h^{-1}) and organic suspended matter (OER, mg h^{-1}). Assuming that absorption of inorganic matter through the digestive system was negligible (Iglesias et al. 1992, Iglesias et al. 1998), the rate of inorganic matter filtration (IFR, mg h^{-1}) was calculated by summing IRR and IER. Thus clearance rates (CR, L h^{-1}) were estimated as CR = IFR/ISM where ISM (mg L^{-1}) was total suspended inorganic matter (Iglesias et al. 1996, Wong and Cheung 1999). By multiplying clearance rates by the total suspended matter (TSM, mg L^{-1}) and by its organic fraction (OSM, mg L^{-1}) total (FR_{TSM}, matter per unit time per mussel, mg TSM h^{-1}) and organic matter filtration rates (FR_{OSM}, mg h^{-1}) were estimated. Ingestion rates of total (IR_{TSM}, mg TSM h^{-1}) and organic matter (IR_{OSM}, mg OSM h^{-1}) were extrapolated as follows: $IR_{TSM} = FR_{TSM} - RR$ and $IR_{OSM} = FR_{OSM} - ORR$, respectively. Absorption food rates (AR, mg h^{-1}) were extrapolated as follows: $AR = IR_{OSM} - OER$, while absorption efficiencies (AE) as $AE = AE/IR_{OSM}$. Preingestive selection efficiencies for the total filtered organic matter (SE_{OSM}) were also estimated according to Navarro et al. (1992).

Chemical Analyses

In the laboratory, samples of water collected in the saltworks and from the experimental filtration chambers, faeces and pseudofaeces were filtered onto pre-washed, precombusted (450 °C, 4 h) and pre-weighed Whatman GF/F filters to determine organic and inorganic matter contents.

All measurements of total organic content in the food (OSM), faeces (FOM), and pseudofaeces (PsOM) were obtained using the difference on ignition method. Filters were weighed after desiccation (105 °C, 24 h) using a Sartorius A200 (accuracy ± 1 μg) and the inorganic fractions of total matter were calculated as the weight of the material remaining after combustion at 450 °C for 4 h. OSM, FOM, and PsOM were calculated from the difference between the weights obtained after desiccation and those obtained after combustion.

For the water samples, the particulate carbohydrate concentrations (P-CHO, mg L^{-1}), particulate proteins (P-PRT, mg L^{-1}), and particulate lipids (P-LIP, mg L^{-1}) were determined using methods reported in Sarà et al. (1998).

LPOM as defined above was also converted into carbon equivalents (using 0.75, 0.40, and 0.49 μg C μg^{-1} conversion factors for particulate lipids, carbohydrates, and proteins, respectively) and used as an expression of the biopolymeric fraction of particulate organic carbon (BPC) (Sarà et al. 1998). Suspended chlorophyll-a (CHL-a) and phaeopigment (PHAEO) concentrations were measured according to Lorenzen and Jeffrey (1980). Pigments were extracted with 90% acetone and phaeopigments determined after acidification with 0.1 N HCl.

The ISM/OSM, LPOM/TSM, and LPOM/OSM ratios (Widdows et al. 1979, Navarro et al. 1993, Navarro and Thompson 1995, Sarà et al. 1998), the C-CHLa/BPC ratio (the concentration of CHL-a converted into carbon units using 52 as the conversion factor, Nival et al. 1972) and the P-PRT/P-CHO ratio (Navarro and Thompson 1995, Sarà et al. 1998) were used as tools for gathering information about the nutritional value of the particulate organic matter. Temperature (T, °C) and salinity (SAL) were measured at each sampling of the saltworks water using a Hydrolab (Inc. Hous-

ton, TX, USA) multiprobe. Salinity signals from the probe were tested monthly using $AgNO_3$ titration.

The relationships between physical, chemical, and trophic variables with physiological changes were assessed using regression and Spearman correlation (r_s) tools (Sokal and Rohlf 1981). Statistica package (Release 5.1; StatSoft, Inc.) was used to perform statistics.

RESULTS

Physical Conditions and Seston Characteristics in the Saltworks

The food composition available to suspension feeders in the study area as a function of different experimental periods is summarized in Table 1. The average temperature was 18.6 ± 7.4 °C, ranging between the minimum measured in December (9.5 °C) and the maximum in August (30 °C). The saltworks waters were consistently hyperhaline, showing SAL ranging between 40 and 53 (average 47 ± 4.3). Average seston concentrations throughout the experimental periods were 81.5 ± 95.5 mg L^{-1}, ranging between an average minimum condition of 10.2 ± 2.7 mg L^{-1} (average of December, January, and June values) and an average maximum condition of 153 ± 86.7 mg L^{-1} (average of March, August, and October values). In the low TSM range, the inorganic fraction greatly exceeded the organic fraction (78% ISM vs 22% OSM), while the situation was completely inverted in the high TSM range, when the organic fraction was more abundant than the inorganic one (97% OSM vs. 3% ISM).

TABLE 1.

Mean values of chemical, physical and trophic variables measured during experimental periods.

Variables	Month					
	Jan	Mar	Jun	Aug	Oct	Dec
T, °C	12.5	15.1	25.0	30.0	20.0	9.5
SAL	58.0	45.0	48.4	53.0	39.6	46.0
CHL-la, μg L^{-1}	0.7	1.7	1.0	0.6	0.8	0.5
PHAEO, μg L^{-1}	0.3	0.9	0.4	0.4	0.3	0.8
TSM, mg L^{-1}	10.0	66.6	13.0	240.0	152.0	7.6
OSM, mg L^{-1}	3.0	63.8	2.7	235.0	146.0	1.3
ISM, mg L^{-1}	7.0	2.7	10.3	4.8	6.3	6.4
ISM/OSM, fraction	2.4	0.0	3.8	0.0	0.0	5.0
OSM/TSM, fraction	0.3	1.0	0.2	1.0	1.0	0.2
LIP, mg L^{-1}	0.1	0.1	0.1	0.1	0.1	0.1
PRT, mg L^{-1}	0.3	0.3	0.3	0.2	0.4	0.2
CHO, mg L^{-1}	0.2	0.1	0.2	0.2	0.2	0.3
PRT/CHO fraction	1.3	2.6	1.4	1.0	1.9	0.6
LPOM, mg L^{-1}	0.6	0.5	0.5	0.6	0.6	0.6
BPC, mg C L^{-1}	0.3	0.2	0.2	0.3	0.3	0.3
C-CHLa/BPC, %	10.2	33.2	16.1	9.8	10.6	7.5
LPOM/TSM, %	5.5	0.7	4.0	0.2	0.4	8.3

Abbreviations: T, water temperature; SAL, water salinity; CHL-a, suspended chlorophyll a; PHAEO, suspended phaeopigments; TSM, total suspended matter; OSM, total suspended organic matter; ISM, total suspended inorganic matter; LIP, lipid concentration in the particulate; PRT, protein concentration in the particulate; CHO, carbohydrate concentration in the particulate; LPOM, labile particulate organic matter as the sum of LIP, PRT and CHO; BPC, biopolymeric fraction of particulate organic carbon calculated by converting LPOM into carbon equivalents—see text for LIP, PRT, and CHO conversion factors; C-CHLa, carbon suspended chlorophyll-a. See text for explanation of ratios.

Phytoplankton biomass was quite low, with mean CHL-a concentrations of 0.88 ± 0.4 μg L^{-1} with a maximum measured in March (1.7 μg L^{-1}) and a minimum in December (0.5 μg L^{-1}). Phaeopigments represented on average 32% of total chloropigments. Chloropigments were almost twofold higher in the high TSM range compared to in the low range (1.1 vs 0.6 μg L^{-1}).

Mean labile fraction concentration (LPOM) of suspended organic matter was 0.55 ± 0.07 μg L^{-1} and throughout the experimental periods represented about 15% of OSM and only 3.2% of TSM. Two conditions to be identified: at low TSM values LPOM represented 29% of OSM, while at high TSM values LPOM represented only 0.45%. Particulate lipids were about 0.07 ± 0.01 mg L^{-1}, representing 13% of LPOM, particulate proteins were 0.27 ± 0.05 mg L^{-1}, representing approximately 49% of LPOM, while carbohydrates were 0.21 ± 0.08 mg L^{-1}, representing about 37% of LPOM. There were no evident differences in lipid concentrations between conditions of low and high TSM concentrations, while proteins were higher (54% LPOM) at high TSM values compared to at low TSM values and, vice versa, carbohydrates were higher in the low TSM range (43%). In the low TSM range, proteins and carbohydrates showed approximately the same concentrations in LPOM producing a P-PRT/P-CHO ratio of about 1, while proteins exceeded carbohydrates twofold in the high TSM range (P-PRT/P-CHO ratio about 2). The unicellular autotrophic fraction represented about 15% of the labile fraction, ranging from 11% in the low TSM range to 18% of LPOM in the high TSM range.

Clearance, Filtration, and Ingestion Rates

The physiological variables estimated in *B. pharaonis* throughout an annual period are summarized in Table 2. Measurements of clearance rate performed using the trace-substance method were compared with values obtained with the biodeposition method. The agreement between the two kinds of estimates is demonstrated by the following equation: $CR_{tracer} = 0.46 \pm 0.71 * CR_{bio}$; $r = 0.95$ (±0.4); $n = 20$; $P < 0.05$ where CR_{trace} was clearance rates measured as the depletion of particle concentration and CR_{bio} was clearance rates measured with the biodeposition method (CR = IFR/ISM; Cranford and Grant 1990, Iglesias et al. 1992, Hawkins et al. 1996). In order to obtain a more precise estimate of feeding response, the average curve of both (trace-substance and biodeposition) was used.

Weight standardized clearance rates were 1.64 ± 0.82 g L^{-1}, ranging between 0.80 ± 0.15 g L^{-1} in June and 3.02 ± 0.11 g L^{-1} in December. Clearance rate appeared to be inversely related to seasonal temperature (Eq. 1; Table 3). High clearance rates (3.02 and 2.1 L h^{-1}) occurred at low temperatures (9 and 12 °C) and minimum CRs at high temperatures (0.80 and 1.1 L h^{-1} at 25 and 30 °C, respectively).

The results of a Spearman correlation analysis between CR and physiological and dietary factors are summarized in Table 4. CR is independent of changes in SAL, while some dietary variables may explain the variability of *B. pharaonis* clearance rates. In particular, CR was negatively correlated with TSM, OSM, CHL-a, particulate proteins, PRT/CHO (Fig. 1b), and CCHL/BPC ratios and positively with ISM/OSM, phaeopigments, particulate lipids, and carbohydrates and LPOM (Fig. 1c).

During the experimental period, *B. pharaonis* filtered on average 110 ± 107 mg h^{-1} of total suspended material (mg TSM h^{-1}), with a maximum in August and October (276 and 262 mg TSM h^{-1}, respectively) and a minimum (10.4 mg TSM h^{-1}) in June.

TABLE 2.

Averaged physiological (n = 84) values estimated in *Brachidontes pharaonis* throughout an annual period in the Stagnone saltworks.

	CR_{DWst}	FR_{TSM}	FR_{OSM}	IRR	ORR	RR	SE_{OSM}	IR_{TSM}	IR_{OSM}	OER	AR	AE
Jan	2.07	20.57	6.10	0.36	0.09	0.45	0.31	20.12	6.01	0.15	5.86	0.97
Mar	1.09	72.47	69.50	0.11	0.67	0.78	0.10	71.70	68.83	0.46	68.37	0.99
Jun	0.80	10.37	2.16	0.68	0.11	0.79	0.33	9.57	2.05	0.43	1.62	0.79
Aug	1.15	276.16	270.68	0.28	0.22	0.50	0.55	275.67	270.46	0.10	270.36	0.99
Oct	1.73	262.76	251.81	0.44	0.15	0.59	0.74	262.17	251.66	11.63	240.03	0.95
Dec	3.02	23.03	3.86	0.38	0.01	0.46	0.89	22.57	3.85	3.60	0.25	0.06
Mean	1.64	110.89	100.68	0.37	0.21	0.60	0.49	110.30	100.48	2.73	97.75	0.79
± SE	0.82	124.77	127.07	0.19	0.24	0.16	0.30	124.80	127.04	4.56	124.97	0.37

Annual means and standard errors for means are also reported.
Abbreviations: (CR_{DWst}, L g h^{-1} = weight standardised clearance rate; FR_{TSM}, mg TSM h^{-1} = total suspended matter filtration rates; FR_{OSM}, mg h^{-1} = suspended organic matter filtration rates; IRR, mg h^{-1} = inorganic content of pseudofaeces; ORR, mg h^{-1} = organic content of pseudofaeces; RR, mg h^{-1} = rejection rates; SE_{OSM} = total filtered organic matter selection efficiencies; IR_{TSM}, mg TSM h^{-1} = ingestion rate of total suspended matter; IR_{OSM}, mg TSM h^{-1} = ingestion rate of total suspended organic matter; OER, mg h^{-1} = organic content of true faeces; AR, mg h^{-1} = food absorption rates; AE, fraction = absorption efifciencies).

FR_{TSM} produced a significant relationship with TSM, which are plotted in Figure 2a and described in Eq. 2 (Table 4). Accordingly, FR_{TSM} increases as a function of TSM up to about 100 mg TSM L^{-1}, after which FR_{TSM} reaches a plateau. Filtration rate was also a decreasing function (Fig. 2b) of labile organic content of suspended material (LPOM/TSM ratio; Table 4; Eq. 3).

Rejection rate was on average 0.60 ± 0.16 mg egested material h^{-1}. The proportion of inorganic egested material was on average 65% throughout the study period. Rejection rate (Fig. 3) reached minimum values in December and January (approx. 0.4 mg h^{-1}) when TSM concentrations were lower (average 8.8 ± 1.6 mg L^{-1}) and ISM/OSM ratios were on average 3.5. RR reached its maximum values in June and March when TSM was below 100 mg L^{-1}, after which RR decreased sharply.

Selection efficiency (SE_{OSM}) ranged between 0.1 (March) and 0.90 (December) (average 0.50 ± 0.22). SE_{OSM} did not show a significant relationship with ISM, while the relationship with LPOM was described by Eq. 4 (Table 3).

Although SE_{OSM} was not measured directly as a function of each food substrate (Urrutia et al. 1996), we tentatively fitted SE_{OSM} versus the different dietary features. CHL-a, carbohydrates, and the P-PRT/P-CHO ratio in the labile particulate organic matter produced significant linear relationships with SE_{OSM}, which are plotted in Figure 4 and described by Eq. 5–7 (Table 3).

According to the above relationships, SE_{OSM} decreased as CHL-a and P-PRT/P-CHO increased, but increased as a function of P-CHO.

TABLE 3.

Relationships between environmental and physiolngical variables in the Sicilian saltworks *Brachidontes pharaonis*.

n.	Equation	r and P
1	$CR = 4.09 - 0.13 \times T$	($r = -0.92$; $P = 0.01$)
2	$FR_{TSM} = -6.1 + 2.1 \times TSM - 0.03 \times TSM^2$	($r = 0.95$; $P < 0.05$)
3	$FR_{TSM} = 17.5 + 495 \times \exp^{(-LPOM/TSM/0.41)}$	($r = -0.91$; $P < 0.05$)
4	$SE_{OSM} = -1.75 + 4.1 \times LPOM$	($r = 0.96$; $P < 0.05$)
5	$SE_{OSM} = 0.92 - 0.49 \times CHL\text{-}a$	($r = -0.78$; $P < 0.1$)
6	$SE_{OSM} = -0.20 + 3.10 \times P\text{-}CHO$	($r = 0.78$; $P < 0.1$)
7	$SE_{OSM} = 0.87 - 0.26 \times P\text{-}PRT/P\text{-}CHO$	($r = -0.72$; $P < 0.05$)
8	$IR_{TSM} = 7.8 + 1.3 \times TSM$	($r = 0.96$; $P < 0.05$)
9	$AE = 0.95 - 37.4 \times \exp^{(-P\ PRT/0.17)}$	($r = 0.93$; $P < 0.05$)
10	$AE = 1.6 - 3.97 \times P\text{-}CHO$	($r = -0.82$; $P < 0.05$)
11	$AR = 2.9 + 1.3 \times OSM$	($r = 0.98$; $P < 0.05$)
12	$AR = -1.24 + 0.98 \times FR_{OSM}$	($r = 0.99$; $P < 0.05$)
13	$AR = -437 + 1812 \times P\text{-}PRT$	($r = 0.87$; $P < 0.05$)

See text for acronyms and explanation. (n = equation number; R = correlation coefficient; P = probability level).

TABLE 4.

Spearman correlation analysis relating standardized clearance rates and physical and dietary measured variables throughout the experimental period.

CR vs.	Rs	P
SAL	0.08	0.47 (ns)
OSM	−0.34	0.001 (**)
ISM	0.16	0.14 (ns)
TSM	−0.49	0.00 (***)
ISM/OSM	0.44	0.00 (***)
CHL-a	−0.58	0.00 (***)
PHAEO	0.31	0.002 (**)
LIP	0.43	0.00 (***)
PRT	−0.25	0.02 (*)
CHO	0.50	0.00 (***)
P-PRT/-P-CHO	−0.50	0.00 (***)
LPOM	0.59	0.00 (***)
LPOM/TSM	0.49	0.00 (***)
C-CHLa/BPC	−0.58	0.00 (***)
LPOM/OSM	0.34	0.001 (**)

[n = 84; (*) = $P \leq 0.05$; (**) = $P \leq 0.01$; (***) = $P \leq 0.001$; (ns) = non-significant difference ($P \geq 0.05$)].
Abbreviations: SAL, water salinity; CHL-a, suspended chlorophyll a; PHAEO, suspended phaeopigments; TSM, total suspended matter; OSM, total suspended organic matter; ISM, total suspended inorganic matter; LIP, lipid concentration in the particulate; PRT, protein concentration in the particulate; CHO, carbohydrate concentration in the particulate; LPOM, labile particulate organic matter as the sum of LIP, PRT and CHO; BPC, biopolymeric fraction of particulate organic carbon calculated by converting LPOM into carbon equivalents—see text for LIP, PRT, and CHO conversion factors; C-CHLa, carbon suspended chlorophyll-a). See text for explanation of ratios.

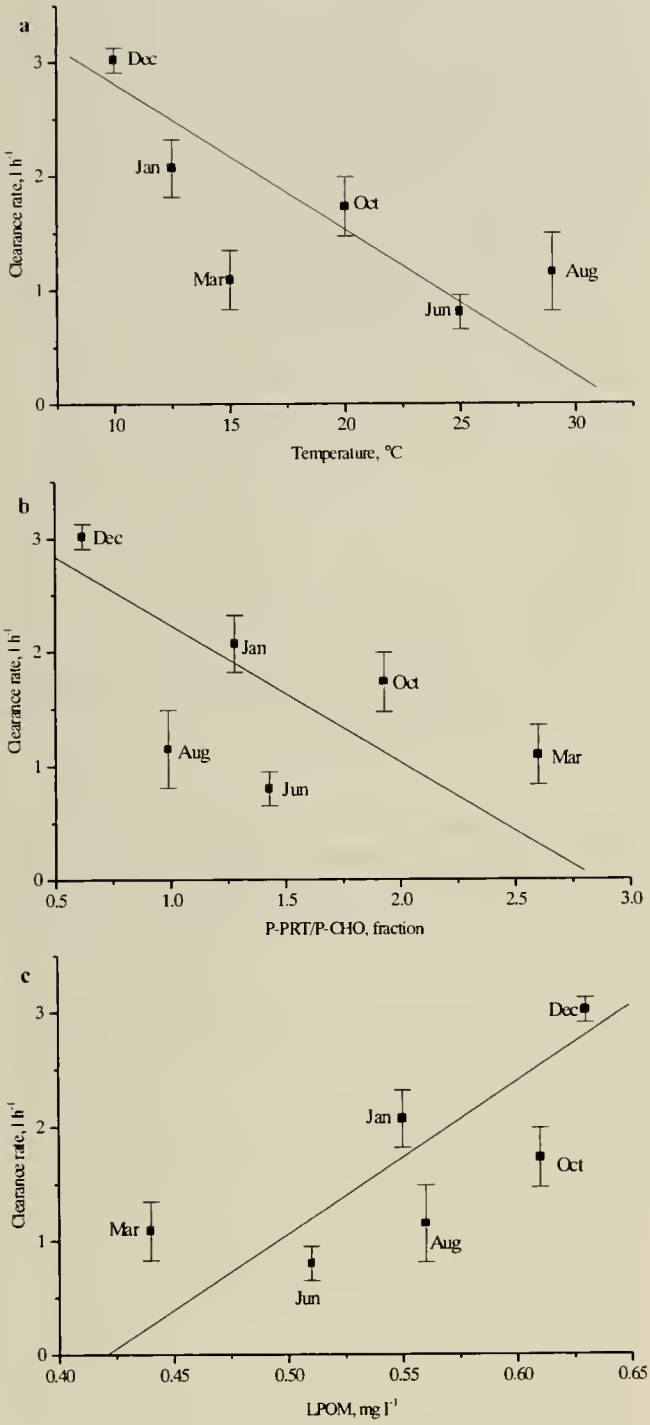

Figure 1. Relationship between a) temperature (T, °C); b) labile particulate organic matter concentrations (LPOM, mg L^{-1}); c) protein by (do you mean 'over' i.e., divided by?) carbohydrate ratio (fraction) and clearance rate (L h^{-1}) as measured throughout the study period.

Total ingestion by the mussels was estimated by subtracting total rejection from total filtered suspended material (IR = FR – RR; Wong and Cheung 1999). The average ingestion rate during the experimental periods was 110.3 ± 124 mg TSM h^{-1}, with a maximum in August (276 mg TSM h^{-1}), and a minimum in June (10 mg TSM h^{-1}). The relationship between rates of seston ingestion (as IR_{TSM}) and TSM is described by Eq. 8 (Table 3).

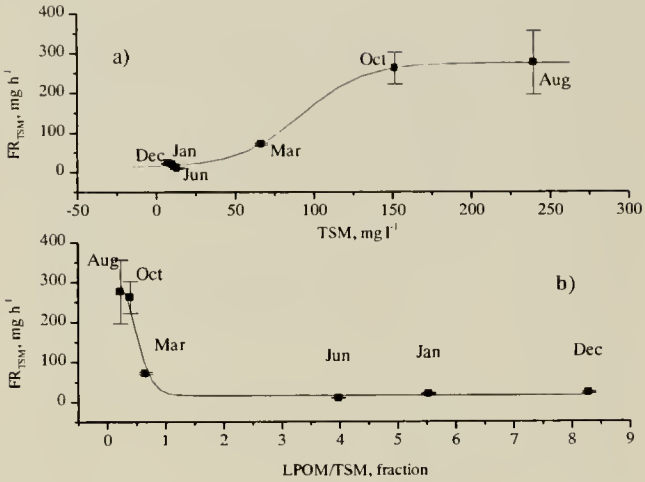

Figure 2. Changes in filtration rates (FR_{OSM}, mg h^{-1}) as a function of a) total suspended material (TSM, mg L^{-1}) and b) labile particulate organic matter concentrations by total suspended material ratio (LPOM/TSM, fraction).

Ingested organic matter ($IR_{OSM,\ mg\ h^{-1}}$) varied between about 2 mg OSM h^{-1} (June) and 270 mg OSM h^{-1} (August). Ingested organic matter presented the same kind of relationships observed between IR_{TSM} and TSM.

Absorption efficiency ranged between 0.1 (December) and 0.99 (March and August). AE increased as a function of ingested organic material (IR_{OSM}; Fig. 5), showing a plateau after approximately 7 mg ingested OSM and as a function of nitrogen content in the available food (P-PRT; Eq. 9; Table 3). Also, AE showed a negative correlation with particulate carbohydrates, described by Eq. 10 (Table 3). Absorbed organic material was at a minimum in December (0.18 mg OSM h^{-1}) and June (1.6 mg OSM h^{-1}), and a maximum in August and October (270 and 240 mg OSM h^{-1}), respectively). Absorption rate was a positive linear function of the amount of seston organics (OSM) and of filtered organic matter (FR_{OSM}; Eq. 11–12; Table 3).

The rate of absorbed organic matter seemed to reach a maximum at 0.2 mg P-CHO L^{-1}, after which a large decrease was observed when P-CHO reached higher concentration values. AR

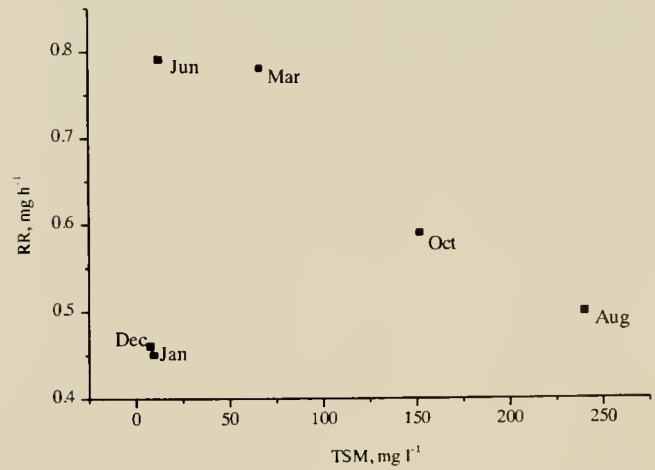

Figure 3. Changes in rejection rates (RR, mg h^{-1}) as a function of total suspended material (TSM, mg L^{-1}).

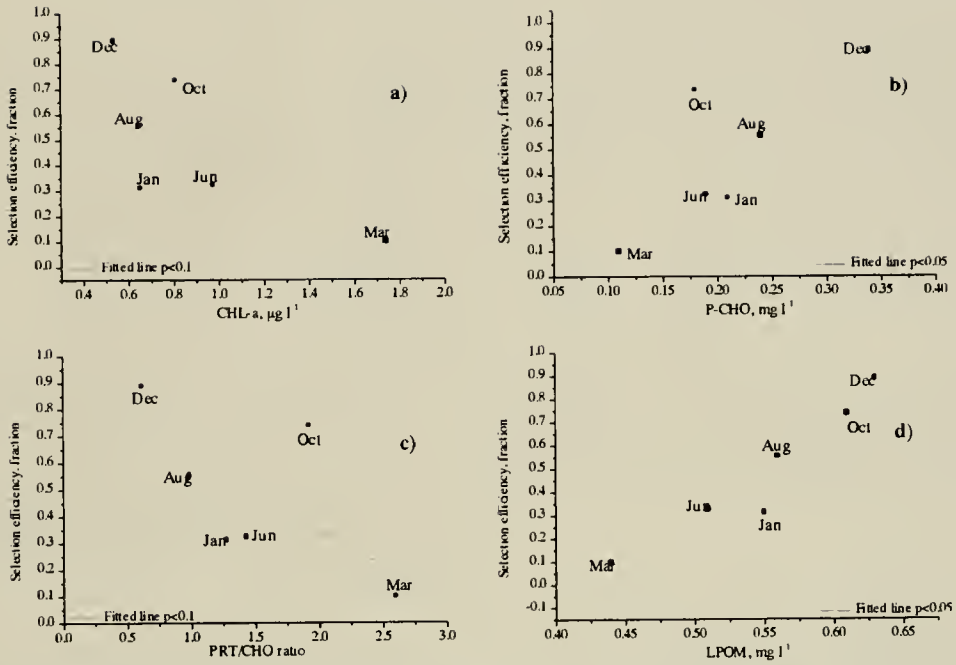

Figure 4. Changes in selection efficiency (SE) as a function of a) chlorophyll-a (µg L^{-1}); b) particulate carbohydrate (mg L^{-1}); c) particulate protein by carbohydrate ratio (fraction); and d) labile particulate organic matter concentrations (LPOM, mg L^{-1}).

seemed to assume a different relationship to particulate proteins (Eq. 13; Table 3).

DISCUSSION

The physiological process of *B. pharaonis* measured *in situ* using the biodeposition method provided a series of complex relationships. Although *B. pharaonis* represents one of the most common species along Red Sea, Pacific, and Indian coasts (Morton 1988), its physiological processes and feeding behavior have never been measured, either in the laboratory or in ambient conditions. Consequently, the general physiological responses of *B. pharaonis* are compared here with those of other species which have been investigated more closely.

The complexity of feeding behavior in *B. pharaonis* appears to be correlated with their complex and varying environmental conditions. Bivalve molluscs are a "species mirror" that describe well the conditions of their colonised environments. *B. pharaonis* is possibly a good example of a descriptor species because, like the other bivalves, it possesses compensatory mechanisms to regulate its behaviour in response to fluctuations in environmental and trophic factors.

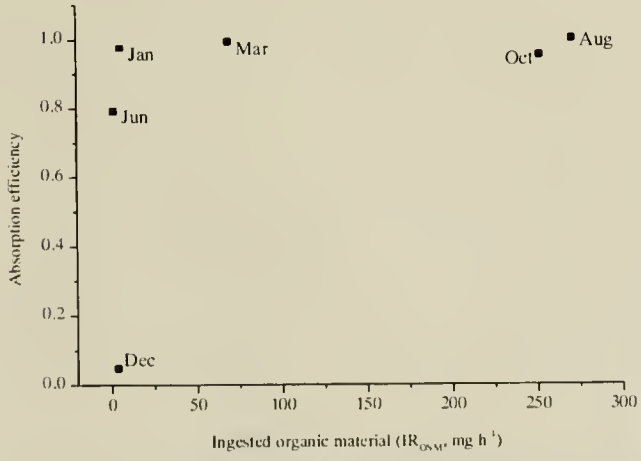

Figure 5. Relationship between ingested organic material and absorption efficiency as measured throughout the study period.

The western Mediterranean saltworks environments, like other similar environments (Pusceddu et al. 1999, Sarà et al. 1999), are characterized by shallowness and partial enclosure, which determine much of the variability in their physical, chemical, and trophical features. A good example is given by water temperature. The temperature of the Mediterranean never falls below 12–14 °C (except in the Adriatic) and above all never exceeds about 26.5 °C (Margalef 1985). Mediterranean benthic communities therefore experience a moderate temperature range throughout the year. In contrast, in the Sicilian saltworks temperature ranges widely, between 9–10 °C in winter and 28–30 °C in summer—a range that is rarely found in other Mediterranean coastal areas (Margalef 1985). These wide variations can be correlated with atmospheric temperature changes and various meteorological phenomena which, in small enclosed shallow basins such as our study area, can strongly influence the usual physical and chemical dynamics (Sarà et al. 1999). Throughout the year, *B. pharaonis* experienced physical conditions that were often close to the survival limits for Mediterranean bivalve molluscs (Sarà et al. 1998). However, it has been documented that *B. pharaonis* is a typical intertidal and hyperhaline species (Por 1971), which can live in very different and quite extreme conditions (Stern and Achituv 1978, Morton 1988).

The average clearance rate (1.64 ± 0.82 L h^{-1}) measured in adults of *B. pharaonis* falls into the general range measured for many other epifaunal bivalve molluscs (Widdows et al. 1979, Riisgård 1988, Navarro et al. 1991, Navarro et al. 1992, Urrutia et al. 1996, Wong and Cheung 1999) and was quite similar to that mea-

sured in other experimental conditions for adults of *B. exustus* (range 1.2–1.7 L h^{-1}; Riisgård 1988).

The first response of *B. pharaonis* to temperature changes seems to be to regulate its clearance rate. The documented response of bivalves to temperature changes is a general reduction in clearance rates at low and high temperatures (Bayne 1976, Jørgensen 1990, Hawkins and Bayne 1992). This has been observed in many bivalve molluscs (Schulte 1975, Aldridge et al. 1995, Hawkins et al. 1998). However, as a general trend, our *Brachidontes* data did not fit with this documented response as its clearance rate showed higher values (2.54 ± 0.67 L h^{-1}; average of December and January values) at the lowest temperatures (10 and 12 °C) and lower CR values with an increase in temperature (up to 25 °C). It is difficult to explain the peak in clearance rate measured in January and December only as a function of temperature. It may be a species-specific response to thermal stress or changes in the endogenous reproductive phases. No literature data regarding similar behaviour in bivalve molluscs exist, and the presence of this species at the low temperatures observed in winter in the study area has not been demonstrated previously. That this species may be better adapted to high than low temperatures (Stern and Achituv 1978) could be explained by the fact that *B. pharaonis* is enzymatically well equipped for bodies of water in which salinity is high and the temperature variable (Stern and Achituv 1978). Further research is needed to resolve the relationship between temperature, CR, and the role of the endogenous reproductive cycles in *B. pharaonis*.

The hyperhaline environment did not seem to produce any effect on clearance, as demonstrated by its independence of salinity. Nevertheless, it has been reported (Bayne 1976, Widdows 1985) that in some conditions salinity can be a regulating factor for clearance rates in several bivalve molluscs, often producing a decrease in clearance rate or, in any case, a certain dependency. However, literature findings refer to acclimatization in typical coastal or estuarine bivalve molluscs, which are generally adapted to sea salinity and are thus less comparable with *Brachidontes*, which are generally well adapted to high salinity (Por 1972, Morton 1988).

The clearance rate of western Mediterranean *B. pharaonis* does not appear to be fundamentally affected by physical factor (i.e., water temperature and salinity) but more likely by fluctuations in the feeding environment. The food supply in the saltworks environment investigated seemed to be limited by resuspension from sediment. The main effect of sediment resuspension is usually an increase in the inorganic fraction of the suspended bulk (i.e., silt material) (Flegey et al. 1992, Sarà et al. 1999, Wong and Cheung 1999). Instead, in the particular conditions of the basin studied (i.e., shallow and enclosed), wind-driven resuspension events (in March, August, and October) led to a marked increase in sedimentary organic matter, producing an unusually high dominance of organic fraction over inorganic (ISM/OSM ratio < 0.1). Although similar results have been observed rarely in coastal environments around the world, the presence of a large amount of rich organic sedimentary detritus composed of seagrass wrack particles and filter feeder biodeposition products may justify organic contents with values that were higher than those for pure phytoplankton cultures.

The nature of the relationships between food acquisition processes in bivalve molluscs and the quantity and quality features of available food has been widely and well documented in the current literature (Widdows et al. 1979, Iglesias et al. 1992, Bayne et al. 1993, Navarro et al. 1993, Navarro and Thompson 1995, Hawkins et al. 1996, Iglesias et al. 1996, Urrutia et al. 1996, Navarro and Widdows 1997, Hawkins et al. 1998, Iglesias et al. 1998, Wong and Cheung 1999). Most of the above-cited articles have shown a general decline in clearance rate with an increase in the quality and quantity of available suspended food (Winter 1978, Bayne et al. 1989, 1993). The present results are consistent with the findings in the literature. Indeed, our data show a general negative dependence of CR on total suspended matter and in particular on its total organic fraction (OSM) (Table 4). Furthermore, the food acquisition process in *Brachidontes* was also dependent on food quality, as highlighted by the relationships between CR and the dietary parameters (Table 4). This relationship has often been tested in laboratory experiments using phytoplankton-based diets. Such experiments have clearly highlighted that most bivalves reduce their clearance rate as a function of an increase in phytoplankton biomass (Winter 1978, Bayne et al. 1989). When the nutritional value of the phytoplankton diet was reduced using inorganic particles to simulate an artificial dilution effect (Iglesias et al. 1992, Bayne et al. 1993), the immediate physiological response was an enhancement of clearance rates. Similar findings have always led researchers to conclude that bivalves respond to food quality. However, these observations cannot be taken as general findings applicable to ambient conditions (Incze et al. 1981, Wildish and Kristmanson 1997) and the necessity to gather information directly from the field has often been highlighted (Bayne et al. 1989, Wildish and Kristmanson 1997).

The CRs obtained in this study were negatively correlated with particulate proteins, suspended cholorophyll-a and consequently with the P-PRT/P-CHO and C-CHL-a/BPC ratios in the particulate, suggesting that *B. pharaonis* reduces its clearance activity as a direct response to an increase in the labile fraction of the available food. In contrast, the positive correlation between CR and particulate carbohydrates and lipids could indicate that in the presence of these classes of compounds *B. pharaonis* increases its rate of clearance to enhance its food uptake. This picture would fit well with the particular feeding environment of *B. pharaonis*, in which the main vegetal component is *Cymodocea nodosa* detritus, which is, by definition (Mann 1988, Velimirov 1991), highly refractory and thus less available to benthic filter feeders (Mann 1988, Valiela 1988). Although this kind of relationship has been hypothesized widely (Bayne 1976, Widdows et al. 1979, Bayne et al. 1993, Dame 1996), it has not been documented clearly.

A diluted effect produced by inorganic material on bivalve clearance rate (see reviews by Hawkins and Bayne 1992 and Dame 1996) has been well verified on many occasions. Consequently, clearance rate increases in correspondence with increments in ISM. However, CRs of saltworks *B. pharaonis* were "statistically independent" of ISM concentrations. Clearance reached its maximum in December and January while under high ISM/OSM ratio conditions but at the lowest temperatures (10 and 12 °C). Such high values cannot be explained by the influence of temperature on CRs but may be explained by the dilution concept widely proposed in the literature (Widdows et al. 1979). However, in order to explain the absence of a well-defined statistical relationship between CRs and inorganic material concentration, we could also hypothesize that in other experimental periods characterized by a large quantity of suspended organics (>60 mg OSM L^{-1}), inorganic matter was not sufficient to reduce the food value. Consequently, *B. pharaonis* did not need to offset the inorganic seston dilution effect by increasing its clearance rate (Dame 1996).

B. pharaonis specimens were able to filter a wide range of total suspended matter (0–280 mg TSM h^{-1}), showing a great capacity to respond to wide changes in food supply. Although our measurements were performed over a wide TSM range, *B. pharaonis* filtering activity was related to seston concentration, like most suspension feeders investigated. The mass of filtered seston increased exponentially up to a certain TSM threshold, after which it reached a plateau followed by a possible decline (Dame 1996). Similar behaviour has been widely observed *in situ* experiments carried out with a seston concentration of up to 100 mg L^{-1} in *Perna viridis* (Hawkins et al. 1998), *Mytilus chilensis* (Navarro and Winter 1982), *Crassostrea belcheri* (Hawkins et al. 1998), *Cerastoderma edule, Mytilus edulis,* and *C. gigas* (Hawkins et al. 1998). Our data would indicate that filtration depends also on the available food quantity (LPOM/TSM, Fig. 2b). When there was a high concentration of LPOM in the saltworks the filtration rate levelled off, whereas when the available organic matter was low the total mass filtered increased. Therefore it is hypothesised that when available food increases *B. pharaonis* levels off its filtration activity because it fulfils its energy requirements with sufficient organic-rich particles.

Our data demonstrate that *B. pharaonis* may not control ingestion rates by varying the rate of pseudofaeces production, and such a mechanism seems to be quite inefficient compared to other bivalve molluscs. Indeed, the average rejection rate by *B. pharaonis* (RR = 0.60 ± 0.16 mg h^{-1}) was lower than those documented for *Mytilus edulis* (1.2 mg h^{-1}, Bayne et al. 1993), *Perna viridis* (0.2–9 mg h^{-1}, Wong and Cheung 1999), *Cerastoderma edule* (2.9 mg h^{-1}; Iglesias et al. 1992) and several tropical bivalves (Hawkins et al. 1998). In most bivalves the control of ingestion level by pseudofaeces production has already been observed (Foster-Smith 1975, Cranford and Gordon 1992, Iglesias et al. 1992, Bayne et al. 1993, Urrutia et al. 1996, Arifin and Bendell-Young 1997, Hawkins et al. 1998, Wong and Cheung 1999) and is considered to be an overflow mechanism. Pseudofaeces production together with the modulation of clearance rate (Winter 1978) can maintain bivalve ingestion rates constant (Iglesias et al. 1992) in order to reduce the "detrimental effect" of food dilution by inorganic material. The material rejected by the saltworks *B. pharaonis* was mainly represented by inorganic material (65%). However, only about 2.2% of filtered seston (77% and 23% of inorganic and organic material, respectively) was rejected as pseudofaeces, indicating that the feeding conditions of the saltworks did not induce a high level of rejection. As confirmation of this, the rejection rates are lower than those reported by Iglesias et al. (1992) for *C. edule*, which rejected material representing approximately 31% of filtered materials (approximately 87% and 13% inorganic and organic material respectively).

However, assuming that ingestion rates were extremely high as a specific response to very high seston organic availability (seston organic content >90%), it would seem that in *B. pharaonis*, there was a lack of ingestion regulation, which, under these trophic conditions would render digestive processes impossible. Without considering physiological compensatory mechanism that have yet to be found in a colonizer mollusc such as *B. pharaonis*, the ingestion rate measured in the saltworks conditions would imply mean gut passage times of about 30–60 min. Such time lengths would generally be incompatible with the breakdown and absorption of food in the mussel gut. We envisage that such discrepancies may be explained by the slight overestimation of ingestion rates due to the use of experimental methodologies (i.e., biodeposition method; Iglesias et al. 1998), which rarely have been tested in similar environments (with huge concentrations of particulate organics), coupled to the great capacity of *B. pharaonis* to adapt to very unusual variability in environmental conditions. On the other hand, the presence of active populations of *B. pharaonis* has also been documented in Mediterranean ultra-oligotrophic sea waters ([CHL-a] $\ll$ 1 μg L^{-1}; salinity 37; Di Geronimo 1971), and such a finding agrees with the huge physiological plasticity of this species.

Under our study conditions, *B. pharaonis* was able to perform sorting of filtered material with an efficiency (SE_{OSM} = 0.5) similar to that reported for other bivalves (Kiørboe et al. 1981, Iglesias et al. 1992, Hawkins et al. 1996, Navarro and Widdows 1997, Hawkins et al. 1998, Wong and Cheung 1999). Although our experiments were not designed to test specifically the selection ability of the pallial organs in *B. pharaonis*, it is apparent that this species is able to sort between refractory and labile food (see Fig. 3). The general picture of selection efficiency in bivalves is that they have an ability to discriminate organic from inorganic material but generally not refractory from labile organics. Nevertheless, the general tendency of *B. pharaonis* is to increase SE_{OSM} when P-CHO in the available food increases (see Fig. 4b and 4c), producing a dilution of the particulate protein nutritional value with CHL-a not exceeding 1 μg L^{-1}. In contrast, when inorganic material did not exceed 4 mg L^{-1}, selection efficiency decreased. The ability of the pallial organs to sort particles of different organic content and to respond to food quality has already been demonstrated in other bivalves. Thus we believe our results may be explained by (1) a species-specific response (Ward and MacDonald 1996, Wong and Cheung 1999) reflecting the huge plasticity of *B. pharaonis* and its ability to colonize a wide variety of tropical, subtropical, and temperature environments; and (2) a particular strategy activated at a local level by *B. pharaonis* in order to minimise the huge variability in the feeding environment.

Absorption efficiency in *Brachidontes* is a complex process that needs further investigation through specifically designed experiments. It has been documented that most bivalve absorption efficiency values are generally low, ranging from 0.4 to 0.6. In the present study, the average AE value of *B. pharaonis* (0.79) was comparable to those reported for other tropical bivalves (Hawkins et al. 1998) but higher than those for *Mytilus edulis* and cockles (Bayne et al. 1989, 1993, Navarro and Widdows 1997, Wong and Cheung 1999). Also, AE depended positively on ingested organic matter (IR_{OSM}), reaching higher values when IR_{OSM} exceeded 6–10 mg h^{-1} and remaining fairly constant above this level.

This type of dependence agrees with that observed in *Perna viridis* and other tropical bivalves (Hawkins et al. 1998, Wong and Cheung 1999), but it differs from those obtained in *Mytilus edulis* (Bayne et al. 1989) and *C. edule* (Iglesias et al. 1992, Urrutia et al. 1996). Moreover, AEs of *Brachidontes* were apparently dependent on the quantity and quality of ingested matter. Significant positive relationships were observed between AEs and particulate proteins, while particulate carbohydrates showed a negative correlation. Indeed, it seemed that absorption efficiency reached higher values when proteins exceeded approximately 0.23 mg L^{-1}, while they decreased when carbohydrates increased. This suggests that *B. pharaonis* may preferentially absorb proteins with respect to carbohydrates and lipids. This hypothetical order of absorption efficiencies contradicts the findings of Bayne et al. (1993), who described higher efficiencies for carbohydrates than for proteins and lipids. However, it agrees with the results of Hawkins and Bayne (1985), who recorded the same rank order of absorption efficiencies for biochemical components of the diet.

This *B. pharaonis* rank order of absorption efficiencies agrees with our unpublished data on suspended bacteria in the saltworks (Sarà, La Rosa and Mazzola, unpublished data). We found concentrations of attached-bacteria fraction in the saltworks water particulate of up to 1×10^9 [two orders higher (10^9 vs. 10^7)] than those found in Mediterranean fish farming impacted coastal waters and sediments (Mazzola et al. 1999, La Rosa et al. in press). This would clearly justify the preferential absorption of proteins by *B. pharaonis*. In addition, the marked dominance of the attached-bacteria fraction in the particulate also indicates that *B. pharaonis* may be able to actively exploit nitrogen from bacteria, as suggested by Langdon and Newell (1990), justifying estimated absorption efficiencies which are so high and so rarely documented in the current literature.

Our findings agree well with the good sorting ability observed in *B. pharaonis*. The hypothesized ability to sort labile (mainly organic compounds originating from bacteria?) from refractory material at the level of the pallial organs may allow *Brachidontes* to maintain constant ingestion rates by channelling more labile (i.e., more digestible) material into the gut. Absorption rate followed the same trend and both the quantity and quality of ingested organic matter was affected. The correlation of absorption rate with filtration activity demonstrates that *B. pharaonis* control of feeding begins with food acquisition processes. Similar findings have been documented in many bivalves, above all in those environments that are limited by high variability in food supply (Iglesias et al. 1992, Bayne et al. 1993, Navarro and Widdows 1997, Wong and Cheung 1999).

CONCLUSIONS

The mechanisms controlling food acquisition processes in *B. pharaonis* appear to reflect the particular feeding environment in which it lives. The complex feeding process of *B. pharaonis* is regulated by the first stages of feeding (clearance and filtration), and most of the control reflects the quantity of food absorbed. In this paper we have clarified the following points:

1. Clearance rate does not appear to be fundamentally regulated by physical factors (i.e., water temperature and salinity), but may reflect fluctuations in the feeding environment and, more closely, the quantity and quality of food available. This finding is consistent with the idea of a trophic regulation of feeding processes in bivalves (Bayne, 1998). However, it contradicts Jørgensen's idea that food uptake in bivalves is an autonomous process which is not regulated at the organism level according to nutritional needs (Jørgensen 1990, Clausen and Riisgård 1996). Further investigation is needed to resolve and fully understand this apparent discrepancy.
2. *Brachidontes* may control ingestion rate by varying the rate of pseudofaeces production, although our data demonstrate that this may not be a very efficient response under the prevailing environmental conditions. However it is able to perform sorting of filtered material. These mechanisms may be used by this species to maintain the ingestion rate constant, as this rate may be affected by the "dilution effect" of both inorganic material and refractory matter in the available food.
3. Absorption efficiencies may be sensitive to the balance of biochemical components in the diet (on average composed of 50% P-PRT, 38% P-CHO and 12% P-LIP). Accordingly, we can infer that *Brachidontes* absorbed organic matter with different efficiencies for proteins, carbohydrates and lipids.

In conclusion, although these findings are currently being investigated further in a set of specifically designed experiments, in the meantime we can say that the *Mediterranean B. pharaonis* seems to be a "complex machine" that operates as a function of a complex synergy of trophic, chemical and physical factors. Such a fact may be the key to understanding the huge plasticity of this organism, which is able to colonize a great variety of habitats at different latitudes around the world.

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KARYOTYPES OF THREE SPECIES OF *CORBICULA* (BIVALVIA: VENEROIDA) IN KOREA

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ABSTRACT The chromosome numbers of three species of Korean *Corbicula* are investigated here: *C. fluminea* 54, *C. papyracea* 54, and *C. colorata* 38. In *C. fluminea* and *C. papyracea*, the mitotic chromosomes from 18 sets of three chromosomes each showed that these two species are triploids. In *C. colorata*, the mitotic chromosomes from 19 groups with two chromosomes each indicated that it is a diploid. *C. fluminea* and *C. papyracea* have one set of metacentric chromosomes, five sets of submetacentrics chromosomes, and 12 sets of subtelocentric chromosomes. *C. colorata* has been considered a subspecies of *C. papyracea*, but its karyotype indicates that it is a distinct species since it is undoubtedly reproductively isolated from the other two Korean species studied.

KEY WORDS: Diploid, triploid, *Corbicula fluminea*, *C. papyracea*, *C. colorata*, echinostome, Korea

INTRODUCTION

The family Corbiculidae is a group of relatively small Asian clams belonging to the bivalve order Veneroida. They live mostly in freshwater, although several species inhabit brackish water. They are a human food item over much of their natural range, and in some areas the extent of their consumption makes them a significant commercial crop. In its native range, the clam is the intermediate host for *Echinostoma lindoensis* trematodes. Since it is sometimes eaten raw or partially cooked, there is often a high local incidence of echinostomiasis in Asia (Sandground and Bonne 1940, Benthem Jutting 1953, Sinclair 1971, Brusca and Brusca 1990). *Corbicula* is often used as food in Korea, and there is no evidence of such a trematode cycle being established. In North America where *Corbicula* have been introduced by human agency and have then spread widely, corbiculid clams are little eaten, but instead have a negative environmental and commercial impact (Britton and Morton 1982, Cherry et al. 1986, Johnson et al. 1986, Macphee 1986, Page et al. 1986, Potter and Liden 1986, Smithson 1986). The pest aspect of *Corbicula* in North America centers on its high reproductive capacity, high growth rate, free-living juvenile stage, and great powers of dispersal (McMahon 1983).

Hermaphroditic freshwater clams in the genus *Corbicula* have been reported to produce non-reductional spermatozoa in Japanese and Taiwanese species compared with somatic DNA content (Komaru et al. 1997). Okamoto and Arimoto (1986) suggested the possibility that *C. leana* reproduces by gynogenesis, i.e., reproduction by parthenogenesis requiring stimulation by a spermatozoan for the activation of the egg. In Korea the freshwater clam *C. fluminea* has been reported to have a special mode of reproduction: it is ovoviviparous, hermaphroditic, and broods its larvae in the inner and/or outer demibranchs (Kwon et al. 1986).

The chromosome numbers of three bivalve species belonging to the Corbiculidae family have been reported previously: *C. leana* (Nadamitsu and Kanai 1978, Okamoto and Arimoto 1986), *C. japonica* and *C. sandai* (Okamoto and Arimoto 1986). The occurrence of triploidy in *C. leana* has been reported (Okamoto and Arimoto 1986).

In Korea, six species of *Corbicula* are recognized (Kwon et al. 1993). (They did not subdivide *Corbicula* into subgenera). Of these six species, we studied the karyotypes of *C. fluminea*, *C. papyracea*, and *C. papyracea colorata*. These taxa live in freshwater and are all hermaphroditic.

MATERIALS AND METHODS

The specimens used in this study were collected in Korea during March 1998 and February 1999, and examined shortly after collection. Twenty-five specimens of *C. papyracea colorata* were collected in the Chungpyung Dam reservoir; 20 specimens of *C. fluminea* and 22 of *C. papyracea* were collected in Lake Uiam in Chunchon. Chromosome preparations were made from gonadal tissues by the air-dry method of Kligerman and Bloom (1977) with minor modification (Park 1994). Gonadal tissues were treated with 0.1 mL of 0.05% colchicine solution and set aside for 20 to 24 h in a moist chamber at room temperature. The treated tissues were dissected and minced with needles in a hypotonic 0.01% NaCl solution. Separated cells were collected by centrifugation at 1,000 rpm for 10 minutes. These cells were fixed in freshly mixed modified Carnoy's fixative (three parts methanol and one part glacial acetic acid). The supernatant was replaced by fresh fixative. The centrifugation (1,000 rpm, 10 minutes) was repeated two more times. A drop of the cell suspension was then pipetted by a microhematocrit capillary tube and dropped onto a clean slideglass pre-cooled at 4 °C. The cells left on the slide were air-dried and then stained for 10 min with 4% Giemsa (Gurr R66) solution made up in 0.1 M phosphate buffer, pH 7.0. The prepared slides were observed under an Olympus (VANOX) microscope with a 100× (n.a. 1.25) oil immersion objective and a 10× ocular.

Morphological features of the chromosomes used to compare karyotypes were the total lengths and the relative lengths of the chromosomes, as well as the positions of their centromeres (primary constrictions). Nomenclature of chromosome morphological types follows Levan et al. (1964).

Voucher specimens of the *Corbicula* species used in this investigation have been placed in the Department of Parasitology, Yonsei University College of Medicine, Korea.

RESULTS

Corbicula fluminea (Muller)

Chromosomes in 28 cells were observed. This species has 54 chromosomes, which can be grouped into 18 sets of three homo-

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logues (Fig. 1). These 18 sets can be divided into one metacentric group, five submetacentric groups and 12 subtelo- or telocentric groups (Fig. 1). The mean total length of all chromosomes was 44.3 ± 2.80 μm. Table 1 shows mean measurements of chromosomes in six cells. Meiotic chromosomes in this species were not observed.

Corbicula papyracea (Heude)

A total of 54 chromosomes was observed in 28 cells. These chromosomes were from 18 sets with three homologues each. These chromosomes can be divided into one group of three metacentric chromosomes, five submetacentric groups of three chromosomes, and 12 groups of three with subtelocentric chromosomes (Fig. 2). The mean total length of the chromosomes was 42.6 ± 0.16 μm. The maximum length of chromosome number 1 was 3.8 μm. Table 2 shows the mean lengths and relative lengths of each chromosome as examined in five cells. Meiotic chromosomes in this species were not observed.

Corbicula colorata (von Martens)

In 25 cells, 38 chromosomes were observed. The karyotype of this species consists of one pair of metacentric chromosomes and 18 pairs of subtelocentric chromosomes (Fig. 3). The mean total chromosome length based on the measurements of five cells was 56.7 ± 0.19 μm (Table 3). The presence of eggs, sperm, and hermaphroditic in this species was observed.

TABLE 1.

Relative lengths and total lengths (micrometers) of chromosomes of *C. fluminea*.*

Chromosome	RL ± SE	TL ± SE	Type
1	8.58 ± 1.04	3.8 ± 0.37	M
2	6.09 ± 0.80	2.7 ± 0.21	SM
3	5.41 ± 0.76	2.4 ± 0.16	SM
4	5.41 ± 0.72	2.4 ± 0.15	SM
5	5.41 ± 0.67	2.4 ± 0.12	SM
6	5.41 ± 0.67	2.4 ± 0.12	SM
7	5.64 ± 0.43	2.5 ± 0.19	ST
8	5.64 ± 0.39	2.5 ± 0.17	ST
9	5.64 ± 0.39	2.5 ± 0.17	ST
10	5.64 ± 0.34	2.5 ± 0.14	ST
11	5.41 ± 0.84	2.4 ± 0.19	ST
12	5.41 ± 0.79	2.4 ± 0.17	ST
13	5.19 ± 0.73	2.3 ± 0.16	ST
14	5.19 ± 0.66	2.3 ± 0.10	ST
15	4.97 ± 0.39	2.2 ± 0.12	ST
16	4.97 ± 0.35	2.2 ± 0.09	ST
17	4.97 ± 0.35	2.2 ± 0.09	ST
18	4.97 ± 0.33	2.2 ± 0.08	ST

* Based on measurement of six karyotyped cells. RL ± SE, relative length of the chromosome, percentage of the total length of the autosomes in diploid; TL, total length of the autosomes in diploid; PM, metacentric; SM, submetacentric; ST, subtelocentric.

DISCUSSION

The Korean clams *C. fluminea* and *C. papyracea* have 54 chromosomes. When the karyotypes of these two species are analyzed the chromosomes fall into 18 sets of three chromosomes each. Therefore, it seems obvious that *C. fluminea* and *C. papyracea* are triploid species.

A Japanese *Corbicula, C. leana*, also had 54 chromosomes that could be classified into 18 sets of homologues, and they indicated

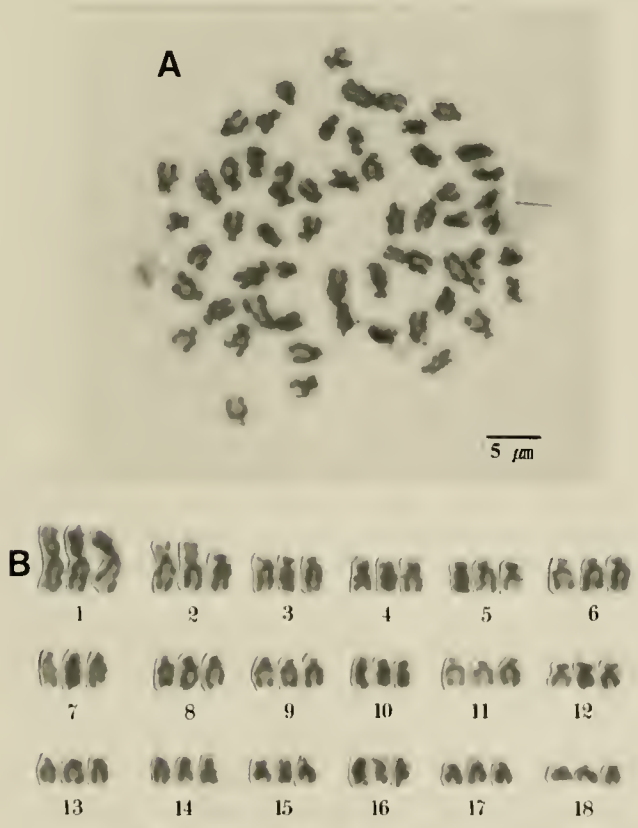

Figure 1. A, Metaphase chromosome of *C. fluminea*; B, Karyotype constructed from A.

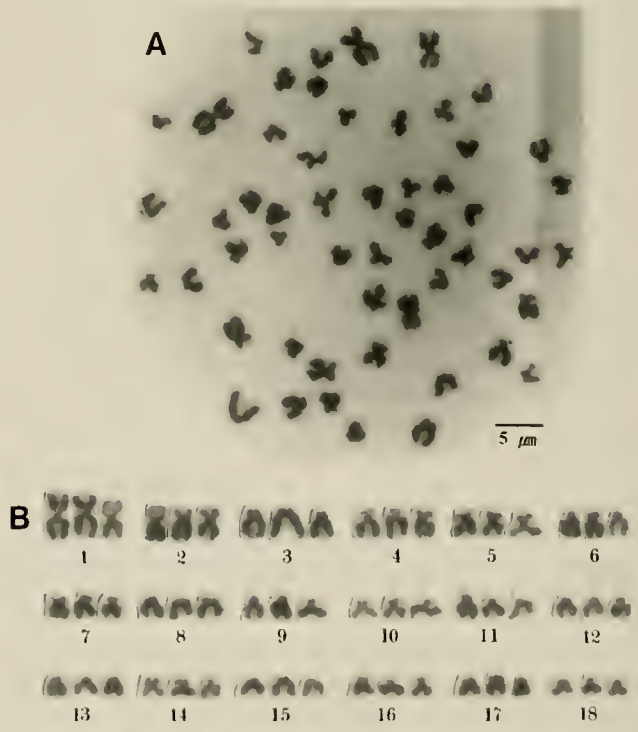

Figure 2. A, Metaphase chromosome of *C. papyracea*; B, Karyotype shown in A.

TABLE 2.

Relative lengths and total lengths micrometers of chromosomes of *C. papyracea*.*

Chromosome	RL ± SE	TL ± SE	Type
1	8.92 ± 0.77	3.8 ± 0.36	M
2	6.10 ± 0.68	2.6 ± 0.23	SM
3	5.63 ± 0.38	2.4 ± 0.17	SM
4	5.63 ± 0.36	2.4 ± 0.16	SM
5	5.40 ± 0.61	2.3 ± 0.17	SM
6	5.40 ± 0.61	2.3 ± 0.17	SM
7	6.10 ± 0.62	2.6 ± 0.20	ST
8	5.40 ± 0.63	2.3 ± 0.18	ST
9	5.40 ± 0.61	2.3 ± 0.17	ST
10	5.40 ± 0.61	2.3 ± 0.17	ST
11	5.40 ± 0.59	2.3 ± 0.16	ST
12	5.40 ± 0.55	2.3 ± 0.14	ST
13	5.16 ± 0.49	2.2 ± 0.13	ST
14	4.93 ± 0.46	2.1 ± 0.11	ST
15	4.93 ± 0.43	2.1 ± 0.09	ST
16	4.93 ± 0.40	2.1 ± 0.07	ST
17	4.93 ± 0.40	2.1 ± 0.07	ST
18	4.93 ± 0.37	2.1 ± 0.05	ST

* Based on measurements of five karyotyped cells.

TABLE 3.

Relative lengths and total lengths (micrometers) of chromosomes of *C. colorata*.*

Chromosome	RL ± SE	TL ± SE	Type
1	6.00 ± 0.64	3.4 ± 0.41	M
2	5.64 ± 0.37	3.2 ± 0.24	ST
3	5.64 ± 0.35	3.2 ± 0.21	ST
4	5.64 ± 0.35	3.2 ± 0.21	ST
5	5.64 ± 0.33	3.2 ± 0.20	ST
6	5.64 ± 0.33	3.2 ± 0.20	ST
7	5.47 ± 0.44	3.1 ± 0.17	ST
8	5.47 ± 0.41	3.1 ± 0.16	ST
9	5.47 ± 0.41	3.1 ± 0.16	ST
10	5.47 ± 0.41	3.1 ± 0.16	ST
11	5.29 ± 0.29	3.0 ± 0.20	ST
12	5.29 ± 0.26	3.0 ± 0.17	ST
13	4.94 ± 0.27	2.8 ± 0.14	ST
14	4.94 ± 0.23	2.8 ± 0.13	ST
15	4.94 ± 0.23	2.8 ± 0.13	ST
16	4.94 ± 0.19	2.8 ± 0.10	ST
17	4.76 ± 0.32	2.7 ± 0.20	ST
18	4.41 ± 0.17	2.5 ± 0.18	ST
19	4.41 ± 0.15	2.5 ± 0.15	ST

* Based on measurements of five karyotyped cells.

that the Japanese species is a triploid. (Okamoto and Arimoto 1986). The second Japanese species had 38 chromosomes, and the third species had 36 chromosomes (Table 4). They concluded that the 36-chromosome species is ancestral to the other two; the 38-chromosome species arose by aneuploidy, and the 54-chromosome species arose by a triploid mechanism. Also, they assumed that the triploid species reproduces by parthenogenesis, the way other triploid animals reproduce (Okamoto and Arimoto 1986). Komaru et al. (1997) reported that hemaphroditic species *C. leana* and *C. fluminia* produce non-reductional spermatozoa compared with chromosome number and DNA content of somatic cells and spermatozoa. On the other hand, the dioecious *C. sandai* has been reported produce reductional spermatozoa. Consequently, they assumed that triploid *C. leana* and diploid *C. fluminea* reproduce by gynogenesis. In this study, chromosome numbers of *C. fluminea* counted 54. We assume that triploid *C. fluminea* in Korea reproduce by parthenogenesis, as Komaru et al. (1997) observed in the *C. leana*.

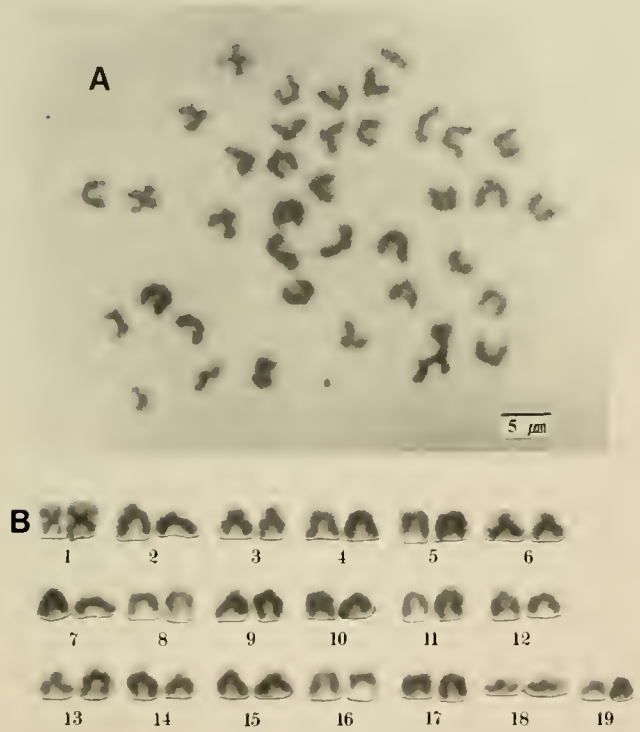

Figure 3. A, Metaphase chromosome of *C. papyracea colorata*; B, Karyotype constructed from A.

In comparing the karyotypes of the Korean and Japanese species, the Japanese triploid (*C. leana*) has one more subterminal-terminal chromosomal homologue and one less submedian homologue. However, because of the minimal difference, including considerable similarity of the chromosomes classified as submedian or subterminal-terminal, this apparent difference between the Korean and Japanese karyotypes may be artificial. The same can be said of the Korean and Japanese diploid species. The Japanese 38-chromosome and 36-chromosome species have a submedian chromosome, whereas the Korean species (*C. colorata*) apparently does not.

Perhaps more significant than the apparent differences in the chromosomal morphologies between the Korean and Japanese species are their similarities (Table 4 and Figs. 1–3), which raises the question of taxonomy. The genus *Corbicula* is well known for its intra- and interpopulational morphological variability, and the resulting over-naming of the "species" has led to much confusion that persists to the present time. Just which nominal species are good biological species needs to be determined. An especially valuable first step in such an endeavor is a cytogenetic one, as demonstrated here.

Finally, it should be pointed out that chromosomes do not support the taxonomic placement of *C. colorata* as a subspecies of *C. papyracea*. The diploid *C. colorata* is obviously reproductively isolated from the triploid *C. papyracea* and thereby the former cannot be considered a subspecies of the latter.

TABLE 4.

Karyotypes in seven species of genus *Corbicula*.

Species	Somatic Chromosomes	Karyotype			References
		M	SM	ST-T	
C. fluminea	54 (3n)	1	5	12	Present study
C. papyracea	54 (3n)	1	5	12	Present study
C. leana	54 (3n)	1	4	13	Okamoto and Arimoto 1986
C. colorata	38 (2n)	1	–	18	Present study
C. japonica	38 (2n)	1	1	17	Okamoto and Arimoto 1986
C. sandai	36 (2n)	1	1	16	Okamoto and Arimoto 1986
"C. leana"	24	–	–	–	Nadamitsu and Kanai 1978

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EVALUATION OF POTENTIAL ANESTHETICS FOR THE FRESHWATER MUSSEL *ELLIPTIO COMPLANATA*

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ABSTRACT A series of experiments was conducted to develop a safe, rapid, and reliable method to relax and anesthetize freshwater mussels for collection of biological samples and assessment of reproductive status. Various concentrations and combinations of eight potential anesthetic agents were administered by bath, slow drip, or injection into the foot or incurrent aperture of 10–40 *Elliptio complanata* per treatment group. Mussels were considered relaxed when the foot extended 2 cm beyond the shell and anesthetized when the valves gaped, and the animal became impervious to touch. Buffered MS-222 (pH 7) produced relaxation within 60 min at concentrations greater than 50 ppm and anesthesia within 3 h at 500–1,000 ppm. Mussels exposed to unbuffered MS-222 at concentrations greater than 250 ppm (pH 3–4) ceased siphoning and closed tightly. Phenoxyethanol at 1.5–3.0% produced anesthesia within 20–50 min but had associated mortality. The effective dose of phenoxyethanol could be reduced to 0.25% with no mortality if mussels were first relaxed with MS-222. Injection of 0.5–5.0 mg succinylcholine chloride into the foot produced rapid immobilization that lasted 20–30 min. Dichlorvos at concentrations of 25–50 ppm induced anesthesia in 3–5 h, but mussels were extremely slow to recover. Clove oil at 0.25–1.00 mL/L anesthetized 65–95% of the mussels tested but proved difficult to work with in confined spaces. Magnesium chloride, potassium chloride, and menthol crystals had no apparent affect on *Elliptio complanata*. This study identified several anesthetic agents for freshwater mussels, each differing in induction time, duration of effect, and degree of foot relaxation. We recommend 500 ppm buffered MS-222 for general laboratory use on *Elliptio complanata* because of ease of handling and safety for both humans and animals.

KEY WORDS: freshwater mussels, *Elliptio complanata*, anesthetics, relaxants

INTRODUCTION

The introduction and rapid spread of the zebra mussel *Dreissena polymorpha* throughout North America has lead to extensive mortality among native unionid mussels in many freshwater systems (Schloesser et al. 1996). Death of the mussels is believed to be caused by either colonization of exposed valves by *Dreissena*, habitat alteration, or depletion of food resources from the water column (Strayer and Smith 1996). No effective means of control or eradication for zebra mussels has yet been devised. One conservation strategy being considered to preserve populations of the most threatened native species is to remove them from *Dreissena*-infested waters physically and place them into uninfected refugia (Cope and Waller 1995). Monitoring of the chosen refugia for adequacy in maintaining health, metabolic condition, and reproduction of the relocated mussels often requires visual examination of mussel soft body parts and collection of tissue and physiological fluids. Samples are usually obtained by mechanically prying the valves apart with a reversing plier, which can fracture the shell, damage the mantle, exhaust the mussel, and tear the adductor. The additional stress of sample collection potentially could reduce the mussels' tolerance to the refugia environment.

An alternate means of obtaining tissue samples is to relax or anesthetize the mussels with a chemical agent. This has been accomplished with such marine bivalves as oysters (Namba et al. 1995, Norton et al. 1996), scallops (Heasman et al. 1995), and giant clams (Rosewater 1963) using benzocaine, chloral hydrate, clove oil, magnesium chloride, menthol, MS-222, 2-phenoxyethanol, propylene phenoxetol, and sodium pentobarbitone. Other mollusks, including sea hares (Beeman 1968), land snails (Chung 1985), pond snails (Girdlestone et al. 1989), and abalone (White et al. 1996) have been successfully relaxed using enflurane, halothane, isoflurane, magnesium sulfate, and succinylcholine chloride. Although not intended as an anesthetic agent, the organophosphate dichlorvos, used for treatment of sea lice in Atlantic salmon net pens, has been reported to relax the adductor muscle of marine bivalves for as long as 42 h after the end of exposure (Le Bris et al. 1995).

Studies with freshwater unionids indicate that combinations of pentobarbitol, urethane, clove oil, MS-222, nembutal, and menthol can induce muscle relaxation or general anesthesia (Araujo et al. 1995, Coney 1993, Smith 1996). These techniques, however, were developed to relax mussels into a lifelike position before death for subsequent dissection or fixation, and thus recovery potential was not considered. The objective of our study was to identify a method to anesthetize freshwater mussels in a relaxed position (i.e., foot extended, shell gaped, unresponsive to touch) in a minimum of time (less than 4 h) using agents that were safe to handle, obtainable without a permit, inexpensive, and provided full and unharmed recovery of the subject animal.

MATERIALS AND METHODS

Twenty-nine separate trials were conducted between July 1995 and June 1997 to evaluate the potential of eight chemical agents to produce nonlethal anesthesia in the freshwater mussel *Elliptio complanata* (Lightfoot 1786). Mature mussels ranging in size from

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63 to 115 mm and 26 to 165 g (mean 95 mm, 100 g) were collected from Pine Creek, Tioga County, Pennsylvania (41°44.408′ N, 077°25.777′ W) and transported in coolers without water to the U.S. Geological Survey (USGS) Northern Appalachian Research Laboratory in Wellsboro, Pennsylvania for experimentation. At the laboratory, mussels were cleaned and measured then sorted into groups of 10 mussels of equal total mass. Each group of 10 mussels was then randomly allocated into a 132-L glass culture aquarium containing 8 cm of white sand substrate and assigned a treatment. The aquaria were each supplied with 1 L/min of 15–17 °C well water and illuminated with overhead fluorescent lamps set to 14 h light:10 h dark photoperiod. Water was circulated within each aquarium by aeration from a 29-cm air diffuser set at the drain end of the tank. Mussels were fed twice daily a mixture of cultured *Nanochloropsis* sp. (Florida Aqua Farms, Inc., Dade City, Florida) and benthic detritus vacuumed from a concrete fish pond. Tanks were cleaned weekly by scrubbing glass, stirring sand, and draining.

Chemicals tested included MS-222 (Finquel; tricaine methanesulfonate, Argent Chemical Laboratories, Redmond, Washington) with or without Tris buffer (Sigma 7–9; Sigma Chemical Company, St. Louis, Missouri), magnesium chloride ($MgCl_2$, Fisher Scientific Company, Fair Lawn, New Jersey), potassium chloride (KCl, Aldrich Chemical Company, Inc., Milwaukee, Wisconsin), succinylcholine chloride, 2-phenoxyethanol, clove oil, menthol (Sigma), and dichlorvos (DDVP; *O,O*-dimethyl-2,2-dichlorovinyl phosphate, AMVAC Chemical Corporation, Los Angeles, California). MS-222, $MgCl_2$, and KCl were dissolved in well water before addition to test tanks. Succinylcholine chloride was dissolved in distilled water then either added directly to the test tanks or injected into the mussel using a 26-G 12.7-mm hypodermic needle. Clove oil and 2-phenoxyethanol were shaken vigorously with 250 mL well water before addition to the tanks. Menthol crystals were powdered and mixed in 100 mL well water before application. Dichlorvos was dissolved in distilled water to form a 6 mg/mL stock solution, and the appropriate volume was added to the tanks.

Tests were conducted by transferring mussels from their culture aquaria into separate treatment aquaria (no substrate) containing 2–30 L well water at the same temperature as the culture tanks. Mussels were arranged within each treatment aquarium in two rows of five and numbered 1–10 according to position. Water was circulated within each aquarium by aeration from a 29-cm air diffuser set in the center of the tank. After a 60 min acclimation, treatments were administered to each aquarium as either a bath (entire dosage applied at one time), by slow drip over a predetermined period, or by injection. Drip treatments were administered from a 1,000-mL separation flask suspended over each tank. Number of replicate tanks of 10 mussels varied from 1–4, depending upon treatment (Tables 1–3). Time to relaxation and/or anesthesia was recorded for each mussel from the moment of treatment application, or from the start of application in the case of drip delivery. Some treatments included a pre-application of unbuffered MS-222 before the primary chemical in an attempt to improve the effectiveness or lower the required dosage of the primary chemical agent. In these cases, time to relaxation was recorded from the MS-222 pretreatment; whereas, time to anesthesia was recorded from application of the primary treatment. A mussel was considered relaxed when the foot extended 2 cm beyond the valve and anesthetized when the valves gaped, and the animal became impervious to touch. These two events are independent phenomena, with the goal being to identify a treatment that produced an anesthetized animal in a relaxed state.

Mussels were removed from treatment tanks either immediately upon detection of anesthesia or after a predetermined period of 1–6 h. Mussels were rinsed in clean water, returned to culture tanks, and arranged in the same order as when in the treatment tank.

Recovery time, defined as the ability to maintain valve closure, was individually recorded from moment of removal from the treatment tank. Mussels were checked 24 h post-treatment for activity (movement or burrowing) and at 7 days for mortality. Mussels subjected to 2-phenoxyethanol, clove oil, menthol, and dichlorvos were also checked for activity 7 days post-treatment (Table 3). Data were analyzed using the general linear models procedure of the Statistical Analysis System (SAS 1988). Any variable expressed as a percentage was arcsine transformed before analysis (Rohlf and Sokal 1981). Differences in treatment means were detected using the Waller/Duncan multiple range test. Orthogonal polynomials were used to make linear, quadratic, and cubic contrasts among treatment effects in the analysis of variance (Rohlf and Sokal 1981).

RESULTS

Bath solutions of 50–250 ppm unbuffered MS-222 produced relaxation in 50–85% of test *Elliptio complanata* within 39–54 min of treatment application (Table 1). Mussels exposed to MS-222 concentrations below 50 ppm siphoned normally but did not respond to treatment; whereas, those exposed to concentrations above 250 ppm stopped siphoning and closed valves tightly within 30 min of treatment application. Water pH decreased within the treatment tanks from pH 7.0 at 50 ppm MS-222 to pH 3.4 at 1,000 ppm MS-222. No bath treatments of unbuffered MS-222 produced anesthesia within the 4-h trial period. Activity at 24 h varied greatly within each group of mussels unrelated to specific treatment. There were no mortalities among the 320 mussels exposed to unbuffered MS-222 bath.

Dripping unbuffered MS-222 to final concentrations of 25–1,000 ppm over 20–120 min produced relaxation in 0–100% of the test animals in times ranging from 24–85 min, depending upon treatment combination (Table 1). As with the bath treatments, MS-222 drip did not produce anesthesia at any concentration tested within the time allowed, and 24-h activity varied greatly among groups. Most mussels exposed to MS-222 concentrations above 250 ppm were tightly closed by the end of the drip period. One mortality occurred among the 320 mussels subjected to unbuffered MS-222 drip, that being at 750 ppm. Injection of 1.2 cc of 1,000 ppm unbuffered MS-222 into the incurrent aperture after 60 min of 100 ppm MS-222 bath produced relaxation similar to bath and drip techniques, but no anesthesia.

MS-222 buffered to pH 7.0 with Tris produced relaxation in 65–95% of the mussels tested within 31–66 min of exposure to concentrations of 50–1,000 ppm (Table 1). Unlike unbuffered MS-222, mussels exposed to buffered MS-222 at concentrations above 250 ppm continued siphoning normally and reached a state of relaxed anesthesia within 126–194 min at concentrations between 500–1,000 ppm (Table 1, Figure 1). Time to anesthesia decreased ($P < 0.01$), and time to recovery increased ($P < 0.01$) with increasing dosage above 500 ppm. Activity at 24 h was lower ($P = 0.03$) among anesthetized mussels than those that were treated but did not reach anesthesia. There were no mortalities among the 260 mussels exposed to buffered MS-222.

TABLE 1.

Summary of trials conducted to evaluate MS-222 as a potential anesthetic for *Elliptio complanata*.

Treatment	Level	Application Method	Mussels #	Mussels Relaxed (%)	Time to Relax (min)	Mussels Anesthetized (%)	Time to Anesthesia (min)	Time to Recover (min)	Active at 24 h (%)	Mortality at 7 days (%)	Notes
MS-222	0 ppm	Bath	20	5	120	0	—	—	40	0	pH 7.1; Trial maxima 4 h
MS-222	5 ppm	Bath	20	15	42	0	—	—	90	0	pH 7.1
MS-222	10 ppm	Bath	20	0	—	0	—	—	75	0	pH 7.1
MS-222	15 ppm	Bath	20	0	—	0	—	—	15	0	pH 7.1
MS-222	20 ppm	Bath	20	0	—	0	—	—	45	0	pH 7.0
MS-222	50 ppm	Bath	40	50	40	0	—	—	32	0	pH 7.0
MS-222	75 ppm	Bath	20	85	54	0	—	—	10	0	pH 6.7
MS-222	100 ppm	Bath	40	85	44	0	—	—	32	0	pH 6.6
MS-222	250 ppm	Bath	40	82	39	0	—	—	48	0	pH 4.5
MS-222	500 ppm	Bath	40	2	205	0	—	—	70	0	pH 3.8
MS-222	750 ppm	Bath	20	0	—	0	—	—	35	0	pH 3.6
MS-222	1,000 ppm	Bath	20	0	—	0	—	—	30	0	pH 3.4
MS-222	0 ppm	Drip—20 min	20	45	112	0	—	—	75	0	Trial maxima 4 h
MS-222	25 ppm	Drip—20 min	20	45	85	0	—	—	90	0	—
MS-222	50 ppm	Drip—20 min	20	85	62	0	—	—	55	0	—
MS-222	75 ppm	Drip—20 min	20	80	46	0	—	—	70	0	—
MS-222	100 ppm	Drip—120 min	20	85	26	0	—	—	50	0	—
MS-222	250 ppm	Drip—30 min	20	30	25	0	—	—	—	0	—
MS-222	400 ppm	Drip—120 min	20	100	30	0	—	—	100	0	—
MS-222	500 ppm	Drip—30 min	20	30	26	0	—	—	—	0	—
MS-222	700 ppm	Drip—120 min	20	0	—	0	—	—	100	0	—
MS-222	750 ppm	Drip—30 min	20	35	24	0	—	—	—	0	—
MS-222	750 ppm	Drip—45 min	20	70	26	0	—	—	30	5	—
MS-222	750 ppm	Drip—60 min	20	90	36	0	—	—	80	0	—
MS-222	750 ppm	Drip—90 min	20	70	42	0	—	—	40	0	—
MS-222	750 ppm	Drip—120 min	20	95	56	0	—	—	60	0	—
MS-222	1,000 ppm	Drip—30 min	20	55	24	0	—	—	—	0	—
MS-222	1,000 ppm	Drip—120 min	20	65	28	0	—	—	100	0	—
MS-222/MS-222	100/1,000 ppm	Bath/inject	20	75	39	0	—	—	40	0	Inject 1.2 cc of 1,000 ppm MS-222 into aperture after 60-min bath
MS-222 Buffered	0 ppm	Bath	20	5	19	0	—	—	70	0	Trial maxima 5 h
MS-222 Buffered	50 ppm	Bath	20	65	50	0	—	—	30	0	All treatments buffered to pH 7.0 with Tris
MS-222 Buffered	75 ppm	Bath	20	70	44	0	—	—	25	0	—
MS-222 Buffered	100 ppm	Bath	40	85	43	0	—	—	30	0	—
MS-222 Buffered	250 ppm	Bath	40	95	31	0	—	—	8	0	—
MS-222 Buffered	500 ppm	Bath	40	80	58	70	194	22	18	0	—
MS-222 Buffered	750 ppm	Bath	40	85	66	85	149	35	5	0	—
MS-222 Buffered	1,000 ppm	Bath	40	88	50	85	126	49	10	0	—

Bath and drip solutions of 10–40 g/L $MgCl_2$ produced some relaxation but no appreciable anesthesia in *Elliptio complanata* (Table 2). Mortality occurred at the higher $MgCl_2$ doses. Mussels subjected to $MgCl_2$ generally closed very tightly within 60 min of exposure and produced copious mucus discharge. Relaxation of the mussels with 100 ppm MS-222 before $MgCl_2$ drip did not help to induce anesthesia. Injection of 30–60 mg of $MgCl_2$ into the incurrent aperture produced an anesthetic state in 10–20% of the mussels, but with 10% associated mortality. The anesthetized mussels, however, were not the same individuals that subsequently died.

Bath solutions of 10–40 g/L KCl had no relaxing or anesthetic effects on *Elliptio complanata* (Table 2). Mussels stopped siphoning upon exposure to KCl and remained tightly closed throughout the 2-h trial. Mortality (5–20%) occurred at all levels of KCl above 10 g/L.

Bath solutions of 250–1,000 ppm succinylcholine chloride had little effect on *Elliptio complanata* (Table 2). Mussels exposed to bath concentrations above 500 ppm became sluggish in response to touch, but only one mussel at 750 ppm reached an anesthetic state. One mussel exposed to 1,000 ppm died within 7 days of treatment. Injection of 0.5–5.0 mg succinylcholine chloride into the foot produced anesthesia in 100% of the mussels within 4–5 min of injection, with recovery in 23–30 min. Mussels gaped and were unresponsive to touch after injection, but feet remained in a constricted unrelaxed state. Mussels injected with 0.5 mg showed some sensitivity to stimulation, but could not sustain valve closure. Activity at 24 h decreased ($P = 0.02$) with increasing dosage. There were no mortalities among the 160 mussels injected with succinylcholine chloride.

Bath solutions of 0.25–3.0% 2-phenoxyethanol induced anesthesia but no foot relaxation (Table 3). Percentage of mussels

TABLE 2.

Summary of trials conducted to evaluate $MgCl_2$, KCl, and succinylcholine-Cl as potential anesthetics for *Elliptio complanata*.

Treatment	Level	Application Method	Mussels #	Mussels Relaxed (%)	Time to Relax (min)	Mussels Anesthetized (%)	Time to Anesthesia (min)	Time to Recover (min)	Active at 24 h (%)	Mortality at 7 days (%)	Notes
$MgCl_2$	0 g/L	Bath	20	10	91	0	—	—	40	0	Trial maxima 2 h
$MgCl_2$	10 g/L	Bath	20	30	11	0	—	—	45	0	—
$MgCl_2$	20 g/L	Bath	20	20	6	0	—	—	25	0	—
$MgCl_2$	30 g/L	Bath	20	0	—	10	29	16	30	5	—
$MgCl_2$	10 g/L	Drip—30 min	10	0	—	0	—	—	60	0	Trial maxima 2 h
$MgCl_2$	20 g/L	Drip—30 min	10	20	14	0	—	—	70	0	—
$MgCl_2$	30 g/L	Drip—30 min	10	20	9	0	—	—	80	0	—
$MgCl_2$	40 g/L	Drip—30 min	10	50	14	0	—	—	30	10	—
MS-222/$MgCl_2$	100 ppm/0 g/L	Bath/drip—90 min	10	80	44	0	—	—	40	0	Trial maxima 4 h
MS-222/$MgCl_2$	100 ppm/10 g/L	Bath/drip—90 min	10	90	37	0	—	—	30	0	MS-222 bath for 60 min before $MgCl_2$ drip
MS-222/$MgCl_2$	100 ppm/20 g/L	Bath/drip—90 min	10	90	37	0	—	—	50	0	—
MS-222/$MgCl_2$	100 ppm/30 g/L	Bath/drip—90 min	10	90	40	0	—	—	10	0	—
MS-222/$MgCl_2$	100 ppm/30 mg	Bath/inject	10	80	61	10	37	15	0	10	Inject 2 cc of 15 g/L $MgCl_2$ into aperture after 90-min MS-222 bath
MS-222/$MgCl_2$	100 ppm/60 mg	Bath/inject	10	50	43	20	48	19	0	10	Inject 2 cc of 30 g/L $MgCl_2$ into aperture after 90-min MS-222 bath
KCl	10 g/L	Bath	20	0	—	0	—	—	55	0	Trial maxima 2 h
KCl	20 g/L	Bath	20	0	—	0	—	—	45	5	—
KCl	30 g/L	Bath	20	0	—	0	—	—	30	5	—
KCl	40 g/L	Bath	20	0	—	0	—	—	35	20	—
Succinylcholine-Cl	250 ppm	Bath	20	5	60	0	—	—	35	0	Trial maxima 2 h
Succinylcholine-Cl	500 ppm	Bath	20	5	60	0	—	—	55	0	—
Succinylcholine-Cl	750 ppm	Bath	20	15	65	5	25	25	55	0	—
Succinylcholine-Cl	1,000 ppm	Bath	20	15	36	0	—	—	45	5	—
MS-222/Succinylcholine-Cl	100 ppm/0.5 mg	Bath/inject	40	55	44	100	4	23	40	0	Inject 0.05 cc into foot after 60-min MS-222 bath
MS-222/Succinylcholine-Cl	100 ppm/1.0 mg	Bath/inject	40	60	54	100	5	27	15	0	—
MS-222/Succinylcholine-Cl	100 ppm/2.5 mg	Bath/inject	40	75	40	100	4	30	2	0	—
MS-222/Succinylcholine-Cl	100 ppm/5.0 mg	Bath/inject	40	65	56	100	4	24	5	0	—

TABLE 3.

Summary of trials conducted to evaluate 2-phenoxyethanol, dichlorvos, menthol, and clove oil as potential anesthetics for *Elliptio complanata*.

Treatment	Level	Application Method	Mussels #	Mussels Relaxed (%)	Time to Relax (min)	Mussels Anesthetized (%)	Time to Anesthesia (min)	Time to Recover (min)	Active at 24 h (%)	Active at 7 days (%)	Mortality at 7 days (%)	Notes
2-Phenoxyethanol	0.25%	Bath	20	10	50	5	88	57	70	—	0	Trial maxima 5 h
2-Phenoxyethanol	0.50%	Bath	20	10	98	25	62	60	50	—	0	—
2-Phenoxyethanol	0.75%	Bath	20	0	—	20	151	28	95	—	0	—
2-Phenoxyethanol	1.00%	Bath	20	5	30	25	104	34	65	—	0	—
2-Phenoxyethanol	1.50%	Bath	20	0	—	60	47	36	40	—	0	—
2-Phenoxyethanol	2.00%	Bath	20	0	—	70	17	64	60	—	5	—
2-Phenoxyethanol	2.50%	Bath	20	0	—	70	32	49	25	—	5	—
2-Phenoxyethanol	3.00%	Bath	20	0	—	75	36	53	20	—	5	—
MS-222/2-Phenoxyethanol	100 ppm/0.25%	Bath/bath	10	80	51	80	43	31	40	100	0	Trial maxima 3 h
MS-222/2-Phenoxyethanol	100 ppm/0.50%	Bath/bath	10	80	47	100	23	48	0	30	0	Add phenoxyethanol after 60-min MS-222 bath
MS-222/2-Phenoxyethanol	100 ppm/0.75%	Bath/bath	10	60	40	100	14	34	10	40	0	—
MS-222/2-Phenoxyethanol	100 ppm/1.00%	Bath/bath	10	90	42	100	18	36	0	30	20	—
MS-222/2-Phenoxyethanol	100 ppm/0.25%	Bath/drip—35 min	20	70	24	35	74	28	35	—	0	Trial maxima 4 h
MS-222/2-Phenoxyethanol	100 ppm/0.50%	Bath/drip—35 min	20	75	28	70	48	22	30	—	0	Drip phenoxyethanol after 60-min MS-222 bath
MS-222/2-Phenoxyethanol	100 ppm/0.75%	Bath/drip—35 min	20	80	28	75	50	28	25	—	0	—
MS-222/2-Phenoxyethanol	100 ppm/1.00%	Bath/drip—35 min	20	60	32	85	40	30	15	—	10	—
Clove oil	0.25 mL/L	Bath	20	10	58	65	79	46	15	60	0	Trial maxima 2 h
Clove oil	0.50 mL/L	Bath	20	15	42	70	68	76	65	75	0	—
Clove oil	0.75 mL/L	Bath	20	0	—	70	56	65	20	95	0	—
Clove oil	1.00 mL/L	Bath	20	25	26	95	57	100	40	80	0	—
MS-222/Clove oil	100 ppm/0.125 mL/L	Bath/bath	20	55	64	55	90	37	15	80	0	Trial maxima 3 h
MS-222/Clove oil	100 ppm/0.250 mL/L	Bath/bath	20	70	62	70	74	46	15	80	0	Add clove oil after 60-min MS-222 bath
MS-222/Clove oil	100 ppm/0.375 mL/L	Bath/bath	20	50	42	85	51	56	15	80	0	—
MS-222/Clove oil	100 ppm/0.500 mL/L	Bath/bath	20	70	56	90	38	59	30	75	0	—
Menthol crystal	125 mg/L	Bath	20	0	—	0	—	—	45	75	0	Trial maxima 3 h
Menthol crystal	250 mg/L	Bath	20	0	—	0	—	—	35	85	0	—
Menthol crystal	375 mg/L	Bath	20	0	—	0	—	—	45	90	0	—
Menthol crystal	500 mg/L	Bath	20	0	—	0	—	—	35	80	0	—
Dichlorvos	0.1 ppm	Bath	20	10	34	0	—	—	65	80	0	Trial maxima 6 h
Dichlorvos	1 ppm	Bath	20	0	—	0	—	—	55	85	0	—
Dichlorvos	5 ppm	Bath	20	10	180	0	—	—	45	55	0	—
Dichlorvos	10 ppm	Bath	20	35	170	0	—	—	40	55	0	—
Dichlorvos	25 ppm	Bath	20	25	184	80	267	24–36 h	0	15	0	—
Dichlorvos	50 ppm	Bath	20	35	108	100	196	24–36 h	0	35	0	—

Figure 1. ***Elliptio complanata*** **relaxed and anesthetized with 750 ppm buffered MS-222.**

anesthetized increased ($P = 0.003$) in a nonlinear fashion (cubic response $P = 0.005$) with increasing dosage to an apparent maximum of about 70% anesthesia at 2.0% 2-phenoxyethanol. Mussels exposed to 2-phenoxyethanol solutions of 1.0% or less continued siphoning throughout the 5-h trial if they did not reach anesthesia; whereas, mussels exposed to concentrations greater than 1.0% closed tightly and ceased siphoning if not anesthetized. Time to anesthesia decreased ($P = 0.005$) with increasing dosage in a nonlinear fashion (cubic response $P = 0.001$) to an apparent minimum at approximately 1.50% 2-phenoxyethanol. Dosage level did not affect time to recovery ($P = 0.30$) nor 24-h activity ($P = 0.16$). Mortality (5% of mussels tested) occurred at 2-phenoxyethanol doses greater than 1.50%. Mussels exposed to 3.0% 2-phenoxyethanol produced copious mucus discharge after being returned to fresh water.

Exposure of mussels to a 60-min bath of 100 ppm MS-222 before 2-phenoxyethanol treatment increased ($P < 0.0001$) anesthetic rate among mussels subjected to low concentrations of 2-phenoxyethanol (Table 3). Time to anesthesia ($P = 0.02$) and time to recovery ($P = 0.04$) were also decreased among these treatment groups by preconditioning with MS-222. However, MS-222 also decreased the dosage level of 2-phenoxyethanol at which mortality occurred to about 1.0%. Bath application of 2-phenoxyethanol was more effective than drip application in inducing anesthesia. Although anesthetized mussels generally constricted their feet while actively exposed to 2-phenoxyethanol, feet became relaxed and extended within a few minutes of return to fresh water.

Bath solutions of 0.25–1.00 mL/L clove oil induced anesthesia in 65–95% of *Elliptio complanata* tested without significant foot relaxation (Table 3). Although anesthetic rate was similar among treatment levels ($P = 0.20$), time to anesthesia decreased (quadratic response $P = 0.006$), and time to recovery increased (linear response $P = 0.01$) with increasing dosage level. Similar to 2-phenoxyethanol, mussels became anesthetized in a constricted position, but relaxed and extended feet upon transfer to fresh water. Exposure of mussels to 100 ppm MS-222 for 60 min before clove oil addition did not increase anesthetic rate at 0.25 mL/L or 0.50 mL/L dosage levels ($P = 0.29$), but did reduce time to anesthesia ($P = 0.03$). There were no mortalities among the 160 mussels exposed to clove oil during these trials.

Powdered menthol crystal had no anesthetic or relaxing effects on *Elliptio complanata* at 125–500 mg/L dosage levels (Table 3). Mussels ceased siphoning upon initial exposure to menthol crystal and remained tightly closed for the duration of the 3-h trial. There were no mortalities associated with exposure to menthol in this trial.

Bath solutions of 0.1–10 ppm dichlorvos had no affect on *Elliptio complanata* within a 6-h exposure period (Table 3). Concentrations of 25–50 ppm produced anesthesia in 80–100% of the mussels tested in an average of 196–267 min. Feet were retracted during anesthesia. Recovery time was prolonged as compared to other treatments, requiring 24–36 h to regain full responsiveness to touch. Mussels anesthetized by dichlorvos were less active ($P = 0.01$) at 7 days post-treatment than mussels that had been exposed to dichlorvos but not anesthetized. Most anesthetized mussels required in excess of 2 weeks to upright themselves and resume burrowing activity. No mortalities occurred among the 120 mussels exposed to dichlorvos during this study.

DISCUSSION

The goal of this study was to identify a nondestructive method to anesthetize unionid bivalves in a relaxed position to allow collection of biological samples and examination of internal anatomy. Relaxation was defined as extension of the foot at least 2 cm beyond the valves and anesthesia as the gaping of valves with unresponsiveness to touch. These two events are separate and unconnected, because a relaxed mussel may be fully responsive to touch; whereas, an anesthetized mussel may be in a constricted position. Because foot extension also occurs with burrowing activity, some control groups were recorded as having relaxation even though no chemical treatment was applied (e.g., MS-222 0 ppm, Table 1). However, foot extension associated with burrowing can often be distinguished from chemical relaxation, because burrowing was a more active process and typically took longer to initiate, usually in excess of 100 min. An exception occurred within the buffered MS-222 control group in which a single animal extended its foot after 19 min. The term "anesthesia" often implies the loss of consciousness or sensitivity, but gaping and lack of response can also be attributable to muscular paralysis. No distinction was made between these two causes of gaping in this study.

Using these definitions, *Elliptio complanata* were relaxed by MS-222 and anesthetized by MS-222, succinylcholine chloride, 2-phenoxyethanol, clove oil, and dichlorvos. MgC_2, KCl, and menthol crystal had no appreciable effect on the mussels. Pretreatment with MS-222 before addition of other primary chemical agents often decreased time to anesthesia and/or dosage requirements for the primary anesthetic and placed the mussels in a relaxed position during anesthesia.

MS-222 (tricaine methanesulfonate) is the most commonly used anesthetic for finfish in North America (Summerfelt and Smith 1990). Mechanism of action is presumed to be through stabilization of cellular membranes in nervous and cardiac tissue, preventing transient increases in sodium permeability and thus decreasing excitability (Letcher 1992). Dosage of 60–250 ppm produces anesthesia in Atlantic halibut, red drum, and goldfish in 3–6 min with recovery in less than 10 min (Malmstrøm et al. 1993, Massee et al. 1995). MS-222 has been used less frequently to anesthetize bivalves. Coney (1993) used 75–100 ppm for 12–36 h, and Araujo et al. (1995) used 500–2,000 ppm for 24 h to relax unionids before lethal fixation. Norton et al. (1996) relaxed the pearl oyster *Pinctada albina* with 1,000 ppm MS-222, but recommended buffering to pH 8 to prevent excess mucus production and

reduce recovery time. Heasman et al. (1995) could not anesthetize the scallop *Pecten fumatus* with 1,000 ppm MS-222 within 60-min exposure.

In this study, buffered MS-222 at concentrations greater than 100 ppm produced foot relaxation in 85% of *Elliptio complanata* within 30–60 min, and concentrations above 500 ppm produced anesthesia within 2–3 h. Mussels further relaxed after transfer to fresh water, but recovered within 30–60 min. Unbuffered MS-222 produced foot relaxation but not anesthesia, presumably because of low pH of the higher dosages. The inability of unbuffered MS-222 to produce anesthesia may not be completely attributed to cessation of siphoning activity, inasmuch as direct injection of 1,000 ppm unbuffered MS-222 into the incurrent aperture also failed to anesthetize the animals (Table 1). Although this may simply indicate that the volume of injected MS-222 was insufficient, it may also indicate that low pH causes physiologic changes in *Elliptio complanata* or chemical changes in MS-222 that affect absorption or metabolism of the compound.

Magnesium chloride solutions of 30–50 g/L have been used to induce valve gaping in such marine bivalves as the Pacific oyster *Crassostrea gigas* (Whyte and Carswell 1983), the European flat oyster *Ostrea edulis* (Culloty and Mulcahy 1992), and the scallop *Pecten fumatus* (Heasman et al. 1995), but not the pearl oyster *Pinctada albina* (Norton et al. 1996). Mechanism of action was considered to be inhibition of muscular contraction attributable to displacement of calcium ions from tissue by magnesium (Whyte and Carswell 1983). In this study, the freshwater mussel *Elliptio complanata* showed some signs of foot relaxation at $MgCl_2$ concentrations exceeding 30 g/L, but remained responsive throughout the 2–4-h trials.

Beeman (1968) reported that injections of 0.05 mg/g of the myoneural blocking agent succinylcholine chloride (ester dimethochloride) produced rapid relaxation of the sea hare *Aplysia californica* with recovery within 45–90 min. The drug was dissolved in seawater and pH of the solution adjusted to 6.4–7.0 with HCl to prevent alkaline hydrolysis of the ester linkage. Chung (1985) found that injections of 0.012 mg/g succinylcholine chloride combined with 2.4 mg/g $MgCl_2$ produced quick and pronounced anesthesia in the land snail *Helix aspersa* with no mortality. In the present study, succinylcholine chloride injections of 0.5–5.0 mg/mussel produced rapid anesthesia (< 5 min) of *Elliptio complanata* with recovery within 30 min. Inasmuch as average live animal mass was 100 g, with about 20% of that being soft tissue, injected dosage was approximately 0.005–0.05 mg/g whole body and 0.025–0.25 mg/g soft tissue weight. All succinylcholine chloride treatments had equal affect on the mussels, except that 24-h activity was depressed at the higher levels. Therefore, smaller dosages than those used in this study would probably be effective in anesthetizing *Elliptio complanata*. MS-222 pretreatment was used to allow easier access to the injection site, and its affects on anesthetic rate are believed to have been minimal.

Propylene phenoxetol and a related compound, 2-phenoxyethanol, have been used to anesthetize a variety of gastropods and bivalves, such as the giant clam *Tridacna maxima* (Rosewater 1963), the abalone *Haliotis midae* (White et al. 1996), and the pearl oyster *Pinctada albina* (Norton et al. 1996). Effective dose of 2-phenoxyethanol was 0.3–0.4% for these species. Heasman et al. (1995) could not relax the scallop *Pecten fumatus* with 0.06% 2-phenoxyethanol and Araujo et al. (1995) could not relax unionid mussels with 1.0% 2-phenoxyethanol solutions. In the present study, *Elliptio complanata* were anesthetized in less than 30 min using 2.0% 2-phenoxyethanol with recovery in about 1 h. This dosage could be reduced to 0.5% or less when combined with an MS-222 pretreatment. Although the sticky, adhesive consistency and noxious fumes of 2-phenoxyethanol made the substance difficult to handle, it produced quicker and deeper anesthesia in *Elliptio complanata* than MS-222 treatment alone. However, exposure to humans may cause irritation to sensitive tissue and damage to kidney and liver, and Summerfelt and Smith (1990) recommend discontinued use as a fish anesthetic because of inherent toxic effects.

Clove oil has been used as a fish anesthetic in Southeast Asia, because it is inexpensive, readily available, and simple to apply. Soto and Burhanuddin (1995) reported that rabbitfish *Siganus lineatus* lost consciousness within 3 min of exposure to 0.1 mL/L clove oil and recovered within 3 min of transfer to fresh water. Araujo et al. (1995) found clove oil to be an effective anesthetic for the freshwater mussel *Unio sp.* and the clam *Pisidium amnicum*, but not for the Asian clam *Corbicula fluminea*. Norton et al. (1996) relaxed the pearl oyster *Pinctada albina* with 1.5 mL/L clove oil. In the present study, *Elliptio complanata* reached anesthesia within 90 min of exposure to 0.125–1.0 mL/L. Lack of dose response in percentage mussels anesthetized may indicate that lower doses of clove oil can be used for relaxing *Elliptio complanata* than were applied in this study. However, clove oil fumes were found to be particularly irritating to the eyes and respiratory tract, and experimentation was discontinued. Use of this substance may be limited to outdoors or to indoors within ventilated hoods.

Menthol has been used as a general anesthetic for invertebrates, because it is readily available, inexpensive, easily handled, and gives acceptable results over a wide range of species (Araujo et al. 1995). Smith (1996) successfully narcotized freshwater mussels by subjecting them to powdered menthol solutions for 24 h. Response, however, is often unpredictable, and neither Coney (1993) nor Araujo et al. (1995) had success using menthol to anesthetize freshwater unionids. Norton et al. (1996) used 250 mg/L menthol crystal to relax the pearl oyster *Pinctada albania*, but in the present experiment *Elliptio complanata* were unaffected by 125–500 mg/L during 3-h trials. Success with menthol may be related to water temperature, considering that Runham et al. (1965) reported improved results by transferring animals to hot water.

Dichlorvos is an organophosphate that affects the nervous system of animals by inhibiting function of the enzyme acetylcholinesterase (Murison et al. 1997). This results in elevated levels of the neurotransmitter acetylcholine, leading to exhaustion and possibly death by continuous neuromuscular stimulation. Bath treatments of 1.0 ppm are used in commercial salmon farms to kill such ectoparasitic crustaceans as sea lice (MacKinnon 1997). Le Bris et al. (1995) found that dichlorvos concentrations of 0.1–1.0 ppm caused adductor muscle relaxation in Manila clams (*Ruditapes philippinarum*) and Japanese oysters (*Crassostrea gigas*) within 2 h of exposure. Recovery occurred within 12 h after removal from treatment with no latent mortalities. In the present experiment, *Elliptio complanata* required much greater dichlorvos concentrations (25–50 ppm) to initiate gaping within the 6-h allotted exposure time. Recovery required more than 24 h, and activity levels remained depressed in excess of 7 days. Thus, dichlorvos would not be appropriate for field use or when immediate recovery and burrowing are required, but may be useful in laboratory situations where extended anesthesia is necessary.

In summary, this study identified several compounds useful in relaxing and/or anesthetizing the freshwater mussel *Elliptio com-*

planata, each with differing induction times, recovery rates, ease of use, and danger to the operator. Our present protocol is to de-water the mussels for 30–60 min before immersion in 500 ppm buffered MS-222. Mussels are taken to near, but not full anesthesia, then held open with either a finger or reversing plier during sample collection and internal examination. Several hundred *Elliptio complanata* were sexed using this technique and held in captivity for over 1 year with no mortality or apparent affect on behavior. MS-222 has also been used to anesthetize *Alasmidonta undulata*, *A. varicosa*, *Lasmigona subviridis*, and *Strophitus undulatus*, although reaction time is quicker for these species than for *Elliptio complanata*. *Pyganodon cataracta* did not respond to 500 ppm MS-222 and may require a different chemical agent and/or technique for anesthesia. Thus, anesthetic protocols will likely need to be developed independently for each new species under investigation.

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SURVIVAL AND GROWTH OF MUSSELS SUBSEQUENT TO HEMOLYMPH SAMPLING FOR DNA

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ABSTRACT With the increasing use of molecular genetic techniques in ecology and evolution, it has become apparent that methods of non-destructive DNA sampling must be developed. In this study we collected 50 blue mussels (*Mytilus* spp.) in each of three size categories: small (10–20 mm), medium (20–30 mm), and large (30+ mm). Hemolymph was extracted from 25 mussels in each size category and the remaining 25 mussels served as controls. The hemolymph was extracted and control mussels were monitored for 384 days, during which time no significant differences in survival or growth were found. We extracted DNA from the hemolymph and successfully polymerase chain reaction-amplified the ITS and Glu-5′ species-specific markers from 81% and 92% of the samples, respectively, and determined that all mussels were *Mytilus trossulus* (Lamarck). The extraction of hemolymph for DNA analysis allows for molecular investigations of populations or species which are either rare or in limited numbers, and for life history investigations where survival of the organism is necessary.

KEY WORDS: non-destructive, *Mytilus*, PCR, DNA, survival, growth, hemolymph

INTRODUCTION

Molecular genetic techniques and the genetic characterization of individuals have become common in the study of the ecology and evolution of marine invertebrates, particularly bivalves (Mitton 1994). The benefit of the polymerase chain reaction (PCR) is that very little DNA is required, thus making analysis possible when the quantity and/or quality of DNA is limited. PCR-based species markers have been used within the *Mytilus* species complex for conservation, ecological, and evolutionary applications (Heath et al. 1995, 1996, Rawson et al. 1996). Molecular genetic characterization has also clarified population genetic structure in a variety of other bivalves (Sarver and Foltz 1993, Manuel et al. 1996, David et al. 1997, Suchanek et al. 1997, Herbinger et al. 1998), as well as aiding in investigations into the ecology and life history of bivalves with planktonic phases (Toro 1998).

Typically, shellfish are destructively sampled in order to sample tissue for DNA extraction. Destructive sampling involves killing the animal to obtain the necessary tissue for genetic analysis (Taberlet et al. 1999). Although this is acceptable for some studies (Sarver and Foltz 1993, Heath et al. 1995, Hare et al. 1996, Heath et al. 1996, Suchanek et al. 1997, Herbinger et al. 1998, Toro 1998), destructive sampling is clearly not acceptable for studies involving growth or survival measurements, or for investigations of small or rare populations. For such studies there is a need for a technique that would allow the collection of DNA without harming the organism. Non-destructive sampling generally involves capturing the target organism, taking an invasive sample without killing it, and then releasing it (Taberlet et al. 1999). It is, therefore, important that any potential technique be tested for even minor adverse effects on the survival or growth of the target organism.

Here we describe sampling hemolymph for DNA extraction from shellfish. Specifically, we sampled hemolymph from three size categories of blue mussels (*Mytilus* spp.), extracted DNA, and amplified fragments using PCR with species-specific markers. Hemolymph is made up of mostly water, but does contain cells, including nucleated hemocytes (Morse and Zardus 1997) and is responsible for the transportation of digestion products throughout the body (Brusca and Brusca 1990), among other functions. We followed the survival and growth of hemolymph-extracted and control mussel groups for over 1 y to ascertain whether this technique resulted in decreased survival and/or growth.

MATERIALS AND METHODS

Fifty mussels in each of three size categories were collected from the western coast of Quadra Island, located near Campbell River, British Columbia, Canada. The mussels were measured with calipers to the nearest 0.01 mm and sorted into small (10–20 mm), medium (20–30 mm), and large (30+ mm) size categories. In each group, hemolymph was extracted (50–200 μL) from 25, while the other 25 (control) mussels were handled, but not sampled. A 1-cc syringe (22-gauge, 1.5-inch needle) was inserted through the rear hinge joint and hemolymph was extracted until no more fluid could be removed. The extracted hemolymph was expelled into 1.0 mL of 95% ethanol and stored at room temperature. After sampling, mussels were placed in six cages (3 hemolymph-extracted and 3 control) and hung approximately 1 m below the surface at the original collection site. The cages were 5 × 5 × 10 cm and were slotted to enable free water flow through the cages, but excluded potential predators.

The hemolymph and alcohol were transported to the laboratory where they were centrifuged (13,000 rpm, 15 min), the liquid was removed, and the pellet dried (LABCONCO Centrivap Concentrator) at 60 °C for 8 min. The dried cells were digested overnight in 200 μL of lysis buffer (10 mM Tris-HCl, pH 8.0, 15 mM ethylenediamine tetra acetate, and 0.5% sodium dodecyl sulphate) and 125 μg of proteinase K at 37 °C. The solution was then extracted once with an equal volume of phenol:chloroform: isoamyl alcohol (24:24:1), followed by isopropanol precipitation (Heath et al. 1995). The extracted DNA was resuspended in 100 μL of double-distilled water and was then PCR-amplified following the ITS protocol described in Heath et al. (1995) and the

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Glu-5′ protocol described in Rawson et al. (1996). Both markers give species-specific results (Heath et al. 1995, Rawson et al. 1996) and thus we are reasonably certain we amplified target DNA, and not contaminating DNA. The PCR products were visualized on a 1.8% agarose gel stained with ethidium bromide (Fig. 1). Individual mussels were scored for genotype at each marker locus on the basis of a diagnostic restriction fragment length polymorphism (ITS: Heath et al. 1995) or using an automated DNA sequencer to determine amplified fragment length (GLU-5′; Rawson et al. 1996).

Between April 3, 1998 and April 22, 1999, the mussels in this experiment were measured and the survivors were counted at three sampling times after transfer to the experimental cages (58, 140, and 384 days). Student's *t* test (shell length) and chi-square (survival) were used for statistical analysis to determine whether differences existed between the two groups at day 58 and day 384. Day 58 comparisons were made to test for short-term effects, while day 384 comparisons were made for long-term effects.

RESULTS

DNA was successfully PCR-amplified from 61 of the 75 samples (81%) for ITS, and from 69 of the 75 samples (92%) for GLU (Fig.1). There was no consistent effect of mussel size (and hence hemolymph volume) on the success of the PCR amplification. All mussels were determined to be *Mytilus trossulus*. The hemolymph technique was found to have little effect on either survival or growth (Fig. 2). At day 58 and day 384, the survivorship of the hemolymph-extracted mussels was not found to be significantly different than the survivorship of the control mussels in any of the size categories ($P > 0.10$). At day 58, the control mussels were slightly larger than the hemolymph-extracted mussels in the small size category ($P = 0.018$), but there was no significant difference in either the large or the medium size categories ($P > 0.50$). At day 384, the hemolymph-extracted mussels were larger than the control mussels in the large size category ($P = 0.033$), but there was no significant difference in the medium or small size categories ($P > 0.10$).

There were also no consistent differences found in survival or growth among the mussel size categories. The smallest mussels we sampled were between 10 and 20 mm and had mortality and growth similar to the larger size categories.

DISCUSSION

We sampled a wide range of sizes of mussels using a nondestructive method of DNA sampling and successfully extracted DNA for PCR purposes from most of the mussels, including those in the small category. Our PCR success rates were comparable to those of Heath et al. (1995) who used destructive tissue sampling methods. The technique described here is a useful tool for fieldwork, as it does not require the killing of the organism under study. Furthermore, we found no consistent effect of hemolymph sampling on either survival or growth of the mussels. Although this is not surprising for the larger mussels, it is unexpected for the small animals, as the extraction of a large portion of the organism's body fluid would be expected to negatively effect the organism's growth and/or survival.

Although we used mussels, our technique is applicable to other bivalve species. For example, Manuel et al. (1996) used a similar technique on scallops (*Placopecten magellanicus*); however, they extracted approximately 5 to 10 times the volume of hemolymph and did not test for potential growth or survival effects of their sampling method. Other researchers have reported hemolymph sampling in bivalves for various purposes, including DNA extraction (Marsh et al. 1995), hemocyte pathology and function (Moore et al. 1991; Oliver & Fisher 1995), and ploidy analysis (Komaru et al. 1988). However, no attempt was made to determine the effect of that sampling on the viability of the animals. Our study also

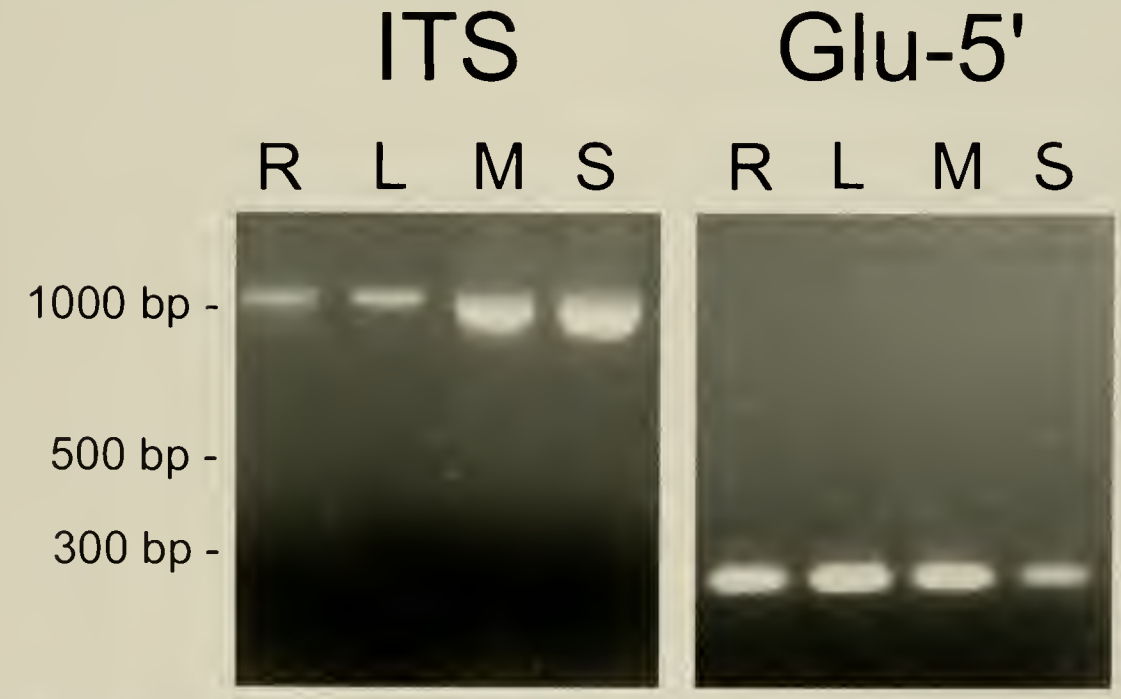

Figure 1. Agarose gel electrophoresis of PCR-amplified DNA fragments using the ITS and Glu-5′ species-specific primer sets. The various lanes are PCR results using DNA from a regular extraction method (R) and DNA extracted from hemolymph taken from mussels in three size classes (L, large; M, medium; S, small).

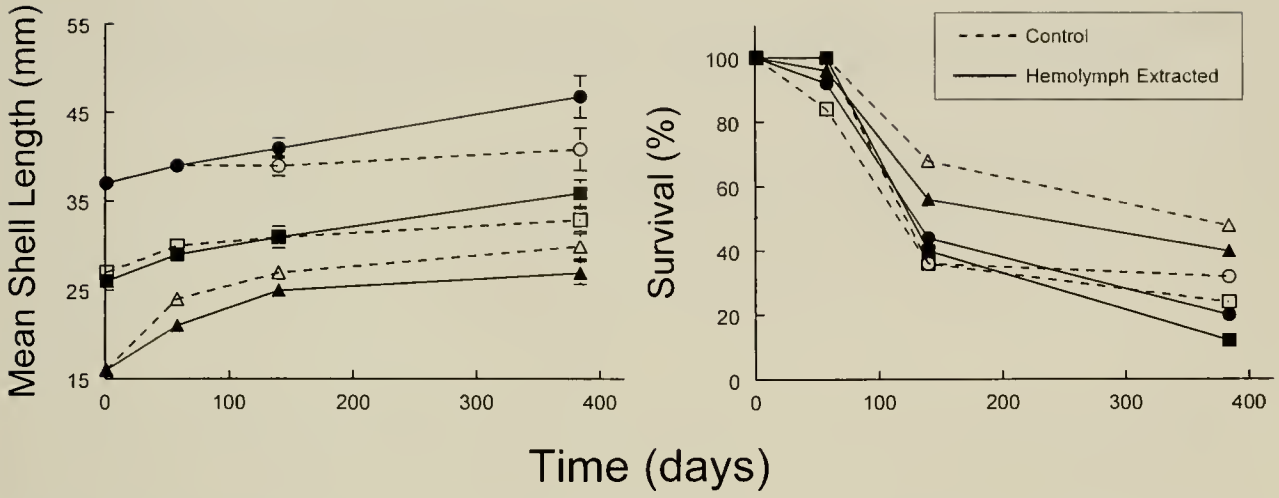

Figure 2. Comparison of mean shell length (±1 SE) and survival for hemolymph-extracted and control mussels from three size classes over a 385-day period, post-treatment. The circles represent the large size class, the squares represent the medium size class, and the triangles represent the small size class. The filled symbols with the solid lines are the hemolymph-extracted mussels, while the open symbols with the dashed lines are the control mussels.

showed the syringe-extracted hemolymph consistently provides PCR-quality DNA from large numbers of animals sampled under field conditions. Hemolymph extraction clearly has considerable potential for studies requiring the non-destructive sampling of DNA from bivalves, and thus has applications for growth and survival studies. This technique will also be useful for studies of the ecology and population dynamics of bivalves where destructive sampling of the organism is either not permitted or not desirable for the experimental design.

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EFFECT OF ARTIFICIAL DIETS ON GROWTH, LIPID UTILIZATION, AND GONAD BIOCHEMISTRY IN THE ADULT SEA URCHIN *PSAMMECHINUS MILIARIS*

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ABSTRACT Three artificial extruded diets of high protein and varying lipid content were manufactured from dried kelp fronds and commercially available raw materials. The diets were fed to replicate groups of *Psammechinus miliaris* (Echinodermata: Echinoidea) over a 14-wk period. Survivorship, weight gain, gonad index, and gonad biochemistry were monitored over the duration of the trial. The diets proved palatable to the urchins and were sufficiently stable in seawater for the urchins to consume in tank-based trials. All of the artificial diets enhanced gonad growth as compared to a reference group fed *Laminaria saccharina* fronds. The satisfactory performance of urchins fed a high-protein diet with a vegetable oil lipid source, composed mainly of oleic and linoleic fatty acids, suggests that *P. miliaris* is able to utilize effectively diets low in polyunsaturated fatty acids (PUFAs). This has positive implications for the manufacture of cost-effective urchin diet by excluding expensive raw materials (i.e., fishmeal, fish oil).

KEY WORDS: Sea urchin, *Psammechinus miliaris*, gonad biochemistry, lipid utilization, artificial diets

INTRODUCTION

The need for a more systematic approach to sea urchin culture has been realized since the early 1980s, when the demand from the French and Japanese markets created a collapse of both their local fisheries and other supplying fisheries; for example, those of the United States, Ireland, Spain, Chile, and the Philippines (McLaughlin et al. 1996; Trinidad Roa 1989, Walker and Lesser 1996).

A concomitant increase in the price of the final product of up to £11.kg^{-1} (raw) in the French market or even up to £83.5.kg^{-1} (processed) in the Japanese market (Grosjean et al. 1998) has led researchers worldwide to a consensus of specific priorities. Among these is to "... determine the nutritional requirements needed to optimize sea urchin gonad growth and whole animal growth. . . ." (Parsons 1997).

Of the three most commonly found edible sea urchin species in the British Isles, *Paracentrotus lividus* (Lamarck), *Echinus esculentus* Linnaeus, and *Psammechinus miliaris* (Gmelin), the latter has been identified as a potential aquaculture species (Kelly et al.1998a). It has an advantage over the other species in terms of roe quality and seems robust in culture (Cook et al.1998, Kelly et al.1998b). Gonad growth can be dramatically enhanced in this species by additional feeding (Cook et al. 1998, Kelly et al.1998b); however, further research is required to produce a refined urchin diet that is cost effective and enhances roe quality. Such a diet should enhance the marketability of the roe in terms of its texture, color, and taste and, therefore, help create a uniform product.

Natural populations of *P. miliaris* are frequently found where the macroalgae *Laminaria saccharina* (L) Lamour are abundant, and they will graze freely on this species of algae. As a food source in a marine environment, fresh kelp fronds are extremely stable. The pronounced seasonal variation in the biochemical composition of kelp (Black 1950), however, would create a serious drawback for using fresh fronds as a year-round food source for sea urchins. In addition, important palatability factors (substances on the algal surface) are likely to be altered or destroyed during preservation and storage (Renaud et al.1990). However, kelp contains large amounts of such structural polysaccharides as alginates, galactans, and galactans mixed with agar or various gums. All these substances act not only as binders but also as gelling agents, and it would, therefore, be potentially advantageous to include dried kelp with commercially available raw materials in the manufacture of a stable, ready-to-use diet for such slow grazing species as *P. miliaris*.

Artificial diets have been used to enhance gonad growth in several echinoid species; however, the amount of biochemical information on the diets provided by the authors varies. Klinger et al. (1986), Lawrence et al. (1989), Lawrence et al. (1992), Lawrence et al. (1997), effectively used artificial diets (consisting of shrimp meal, fishmeal, seaweed, wheat gluten, corn oil, fish oil, corn starch) to evaluate the effect of diet composition on physiological indices (feeding rates, absorption-assimilation, gonadal growth, somatic growth) in *Lytechinus variegatus* (Lamarck), *Loxechinus albus*, and *Paracentrotus lividus*. They provided information on diet composition (percentage of raw materials used) but not on the quality of these raw materials. Nagai and Kaneco (1975) provided a more precise biochemical analysis of the artificial diet they used; however, 37.9% of the proximate composition was not determined. deJong-Westman et al. (1995) did not give the full proximate analyses of the diets used but provided limited biochemical information on the quality of raw materials. These authors also comment that further research is required to refine their diets to the optimum levels of nutrients needed.

Fernandez (1996) compared the performance of the sea urchin *P. lividus* fed three diets made from commercial raw materials (corn flower, wheat, fishmeal, fish oil, sunflower oil) embedded in 12% gelatin solution. Although biochemical analyses for each diet were given, the nutrient levels (energy, protein, lipid, Nitrogen

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Free Extracts [NFE], and oil source) varied in all three of the diets. Therefore, it is difficult to identify which nutrients were responsible for the enhanced growth rates.

Rapid gonad growth has been observed in *P. miliaris* suspended in salmon cages, where salmon feed pellets contributed to their diet, and in tank-based experiments where urchins were fed exclusively on salmon pellets (Cook et al. 1998, Kelly et al. 1998b). However, the high cost of salmon feed dictates that it will never be an economically viable urchin diet, unless the urchins are obtaining it as a by-product of another system where it would otherwise have been lost; for example, in polyculture with salmon. Therefore, the need remains for a cost-effective alternative diet for urchins produced in monoculture and as a preharvest diet for urchins of variable roe quality.

The aim of this study was to design and manufacture a diet for the sea urchin *P. miliaris* in which the lipid content was not based on expensive animal origin oils and to evaluate the performance of urchins fed these diets. Diets were designed with varying lipid level, and a detailed biochemical profile of the diets and the gonads of the urchins was then made with the aim of providing a better understanding of the nutritional requirements of this species.

MATERIALS AND METHODS

Three experimental diets of equal protein but varied lipid content were formulated (Table 1) using prairie meal (gluten), yeast, dried skimmed milk, corn, molasses, dried kelp fronds (*Laminaria hyperborea*, Fosl.), full fat soya, wheat, corn oil, and soya oil. The diets were manufactured using extrusion technology to ensure better adsorption efficiency of the incorporated lipids. The biochemical composition and energy content of the diets was confirmed by the methods described below.

Four hundred *P. miliaris* were collected from local wild populations by SCUBA divers. Urchins [mean horizontal test (shell) diameter 25.76 mm (SD 0.93) and mean initial weight 6.062 g (SD 0.525)] were placed in "NorthWest" perforated plastic trays (0.5 × 0.5 × 0.1 m), which were then placed in rectangular seawater aquaria (3 × 0.65 × 0.65 m) with a flow rate 15 L h^{-1}. One of three artificial diet treatments or a reference diet (*L. saccharina*) was assigned to each tray following the randomized block experimental design (Woolf 1968). There were three (3) replicate groups of urchins for each diet type. The average stocking density per tray was 0.64 kg. M^{-2} (SD 0.016, n = 21–23). Mean salinity recorded over the experimental period was 34.4 ‰ (SD 0.48) and the mean oxygen concentration was 9.27 mg L^{-1} (SD 0.29). Photoperiod was kept constant at 10h: 14 h L: day.

The experiment was conducted from August to December 1997. The urchins were fed once daily, ensuring a pellet of food was available for each individual. The reference treatment was fed *L. saccharina* fronds *ad libitum*. Before each feed, any unconsumed food on the tray and feces underneath each tray were collected by siphoning and were dried until a constant dry weight was achieved. Food consumption was estimated over the first 39 days of the experiment by calculating the difference between the dry weight of food consumed and the dry weight of uneaten food. This was expressed as a percentage of dry food consumed in g live $weight^{-1}$ day^{-1}, where live weight was the average between Day 1 and Day 39 for each replicate treatment.

The urchins were sampled four times throughout the experimental period. Before sampling, the urchins were starved for 48 h and then blotted, and the weight and diameter of each was recorded. At Day 1, Day 39, and Day 70, 10% of the urchins in each treatment were dissected, and the wet weight of their gonads and eviscerated test were recorded. At Day 97, all of the remaining urchins were euthanized, and gonad weight and eviscerated test weight were recorded.

The performance of the urchins was based on the following parameters:

(1) survival rate: 100 × [number of individuals at the end of the experiment/(number of individuals at the beginning of the experiment —total number of individuals euthanized)]; and
(2) gonad index (GI): 100 × [gonad wet weight/total wet weight] (Lawrence et al. 1965).

At the end of the experimental period, the biochemical composition of the gonads of the urchins from the different artificial diets treatments were compared by evaluating the levels of crude protein, crude lipid, glycogen, ash, gross energy, and fatty acid profile. On dissection, the color of the gonads was assessed immediately by matching it to the closest representative color in the Pantone® collection of color standards. The gonad color was always assessed by the same observer, in natural daylight. In addition, on dissection, a portion of gonad from a sample of urchins was preserved in Bouins fluid and embedded in wax. The samples were then sectioned and stained using hematoxylin and eosin for microscopical confirmation of the reproductive stage (Byrne 1990).

Nested analysis of variance (ANOVA), following tests for normality and homogeneity of variance, was employed to examine the significance of differences in the various indices recorded. Percentage data were arcsine transformed (Zar 1996). Where data did not conform to the assumptions of ANOVA, the multiple range Duncan test and the Student-Newman-Keuls test were employed and gave similar results. Statistical evaluation of the results was

TABLE 1.

Proximate analysis of the formulated diets and *Laminaria saccharina* (% on a dry matter basis).

	Diet 1	Diet 2	Diet 3	*L. saccharina*
Crude protein	36.96^{a} (1.02)	37.66^{a} (1.05)	36.05^{a} (0.89)	8.69^{b} (0.82)
Crude lipid	5.10^{a} (0.4)	6.83^{b} (0.3)	9.18^{c} (0.2)	2.57^{d} (0.35)
Crude fiber	2.15^{a} (0.2)	2.01^{a} (0.1)	2.46^{a} (0.15)	7.23^{b} (0.85)
Ash	7.93^{a} (0.5)	7.02^{a} (0.65)	7.95^{a} (0.47)	28.11^{b} (1.05)
NFE*	47.84^{c} (0.45)	46.44^{b} (0.7)	44.34^{a} (0.6)	53.4^{d} (1.35)
Energy in kJ/g^{-1}	9.67^{a} (2.05)	11.37^{a} (2.96)	10.93^{a} (2.72)	12.97^{b} (0.56)

* Nitrogen-free extracts.

Standard deviation in parenthesis (n = 3). Numbers in the same row and with the same superscript are not significantly different ($P < 0.05$).

performed using the statistical package SPSS for Windows (Release 6.1.3).

Analytical Methods

Diets

Before analyses, diet samples were ground with a mortar and pestle until they would pass through a 1-mm sieve. Moisture content was determined by oven drying at 135 °C for 2 h. Analyses for total nitrogen, crude fiber, crude lipid, ash, and gross energy were performed on a dry matter basis and in triplicate. The total nitrogen content of the raw materials was estimated using the Kjeldahl method (Tecator-Kjeltec Auto Analyzer 1030). To convert the determined nitrogen into crude protein, the nitrogen value was multiplied by 6.25, assuming that protein is composed of 16% nitrogen. The percentage of crude fiber was determined (Tecator Fibertec System M / 1020 Hot Extractor) as the difference between the dried insoluble material (remaining after the acid and alkaline hydrolysis) and its respective ashed inorganic fraction. The crude lipid of diets was determined after extraction with petroleum spirit (bp 40–60 °C). Ash was determined by incineration in a muffle furnace at 600 °C for 2 h, and carbohydrates were estimated by subtraction as nitrogen-free extracts (NFE). Energy was determined by chemical oxidation as originally described by O'Shea and Maguire (1962) using potassium dichromate as the oxidizing agent.

Urchin Tissues

Gonad analyses were done in triplicate from a pooled sample of all the urchins within the same replicate. Total nitrogen, ash, and gross energy were estimated as described above. The total lipid content was determined by inserting a known quantity of external standard (tricosanoic acid) during the preparation of fatty acid methyl esters (FAMEs) and quantifying the total quantity of FAMEs based on the results of gas–liquid chromatography. Glycogen was determined by the method of the anthrone reagent after Good et al. (1933) as modified by Seifter et al. (1950) and Hassid and Abraham (1957). The final quantity of determined glucose was divided by the factor 1.11 to convert to glycogen (Morris 1948). Test (shell) ash was determined as previously described for diets.

Fatty Acid Methyl Esters

FAMEs were prepared by the acid esterification method (Christie 1982) after extraction by the method of Folch et al. (1957). The resulting methyl esters were purified using 20 × 20 cm thin layer chromatography (TLC) silica gel G plates with hexane: diethyl ether: acetic acid (90:10:1 v/v) as developing solvent and identified by comparison with known standards on a Carlo Erba gas–liquid chromatograph equipped with a CP-WAX 58 CB (0.2 m) capillary column (25 × 0.25 mm). A thermal gradient of 4 °C/min^{-1} between 160–240 °C was used. FAME identity was confirmed where possible by gas chromatography-mass spectrometry (GCMS) (Thermo Quest / Finnigan, Trace 2000 series).

RESULTS

The artificial diets were palatable to the urchins, and after a period of acclimatization to the new diet format, they fed freely. Once in contact with a pellet, the urchins covered the pellet keeping it in contact with their oral surface and grazed slowly from it. Although stability tests were not performed (Caltagirone et al. 1991), the formulated pellets were stable enough that the urchins

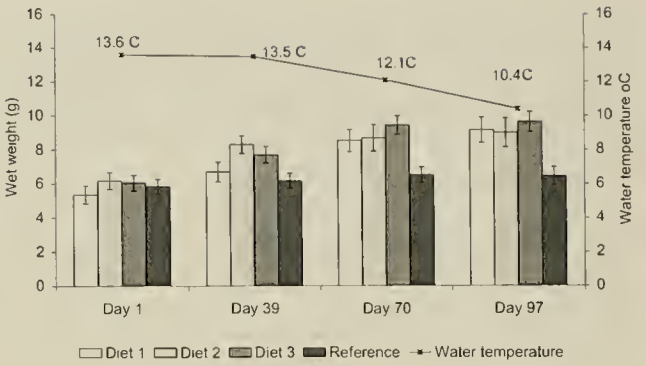

Figure. 1. Live weight of sea urchins (g) fed artificial or reference diets (error bars represent 95% confidence limits).

in trays could continue feeding on one pellet for 24–48 h. Feed consumption (arcsine transformed values) was not statistically different (P = 0.44, F = 0.98, df = 3,10) among treatments. Observed, nontransformed consumption rates were Diet 1 = 1.20% ± 0.77 SD, Diet 2 = 1.74% ± 0.54 SD, Diet 3 = 1.36% ± 0.46 SD, Reference group = 1.08% ± 0.15 SD.

Survival rates were high and not statistically different (P < 0.05) among the groups fed the artificial diets: 93.5% ± 0.5 SD, 94.38% ± 0.58 SD, and 97.83% ± 0.6 SD for the sea urchins fed Diets 1, 2, and 3, respectively, but were significantly higher than that of the reference group (77.95% ± 0.54 SD). This level of survivorship is within the range quoted by Cook et al. (1998) for adult *P. miliaris* (21.3–21.7 mm test diameter) fed *L. saccharina* (80.3%) and salmon pellets (60.3%), respectively.

There was a statistically significant increase in total live weight of all urchins fed the artificial diets overtime (Fig. 1), but no such change was recorded for the live weight of the reference group. The mean test diameter recorded at the beginning of the experiment 25.58 ± 0.55 SD (n = 67–92) was not statistically different from the final mean diameter of urchins from all treatments 24.79 ± 0.81 SD (n = 33–75). All three artificial diets significantly and rapidly enhanced gonad growth (Fig. 2). The GI of all urchins on experimental diets increased from an initial mean of 2.47 ± 1.38 SD to a final mean of 17.33 ± 5.72 SD. On day 70, urchins fed Diet 3 had a significantly higher GI than those fed Diet 1 (P = 0.03, F = 4.27, df 2, 18). However, by the end of the experiment, there

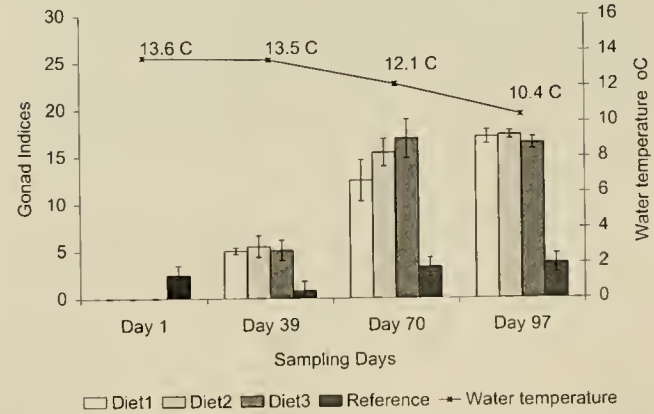

Figure. 2. Gonad indices (GI) of sea urchins (error bars represent 95% confidence limits). Single bar (Day 1) represents mean GI of 10% of the initial population.

were no significant differences in GI of the urchins fed the artificial diets. The recorded increase in GI was equivalent to a mean increase of 1.52 g in gonad weight, and this accounted for most of the recorded wet weight gain (Table 2). There was no statistically significant increase in the GI of the reference group over the experimental period.

Test ash increased from an initial value of 51.98% ± 5.03 SD (n = 8) on day 1 to a mean value of 86.7% ± 0.84 SD (n = 4) on day 39, there were no significant differences between treatments or between the treatments and reference group, although the reference diet, *L. saccharina*, had a higher ash content. Test ash values (of all the treatments) remained high until the end of the experiment (88.75% ± 0.32 SD /n = 10 of dried test).

The artificial diets also had a beneficial effect on gonad color. On day 1, the gonad color of the dissected urchins was gray or brown (Pantone colors 139U, 146U, and 1535U) and classified as unmarketable colors. Feeding a uniform substrate, such as the artificial diets, seemed to limit the range of gonad colors produced. Based on the Pantone collection of color standards, marketable colors were classified as 155U, 156U, 157U, 714U, 1555U, peach and pale yellow. At the end of the trial, percentages of marketable colors were 67.4% for Diet 1, 65.12% for Diet 2, and 59.09% for Diet 3, respectively.

Histological examination of the sectioned gonads at the end of the experiment revealed the higher GIs observed in the urchins fed the artificial diets was attributable to an increase in the nutritive phagocytes, the storage cells in the gonad and not attributable to the presence of developing gametes. The stage of gamete development in the gonad of both the males and females appeared typical of the postspawning recovery phase (Byrne 1990). The urchins seemed to be following the typical pattern of the annual reproductive cycle in *P. miliaris* (Kelly et al. 1998b, Kelly 2000).

Gonad Biochemistry

The biochemical analyses (Table 3) of the gonads of the urchins at the end of the experiment (Day 97) revealed that the reference group had significantly higher proportion of total lipid in the gonad than the urchins fed the artificial diets. However, the total quantity of gonadal lipid accumulated in the reference group was not higher than the rest of treatments as, at that time, the GI (indicating relative gonad size) of the reference group (Fig. 2) was significantly lower than that of the urchins fed artificial diets.

There is no obvious relationship between the amount of lipid in each diet (as the mean of three determinations) and the lipid content of gonads from each treatment (Tables 1 and 3). In addition, there was a surprising variation in the gonad lipid content between replicate samples of urchins fed the same diet. For example, Diet 1 gave gonadal lipid contents ranging from 4 to 17% (on a live weight basis). There is no satisfactory rationalization of this result.

There was no significant difference in gonad protein levels among urchins fed artificial diets or between those fed artificial diets and the reference group. Urchins also accumulated equal levels of energy (per gonad unit weight) when fed the artificial diets, but those fed diet 3 accumulated significantly more than the reference group (Table 3).

Although differing in lipid content, all three artificial diets shared similar lipid sources and, therefore, had similar fatty acid profiles. In turn, this led to similar fatty acid profiles in the gonads. Table 4 shows the mean level (%) and standard deviation for each fatty acid for all the artificial diets (mean of duplicate determinations from each diet) and for the gonads (mean of triplicate determinations from each treatment).

The dietary lipids had a simple vegetable source dominated by 16:0 (palmitic), 18:0 (stearic), 18:1n-9 (oleic), 18:2n-6 (linoleic), and 18:3n-3 (linolenic) acids. The gonads showed reduced amounts of each of these but significantly increased amounts of their elongation-desaturation products 20:1*, (double bond position not determined), 20:2* (possibly one or more non-methylene-interrupted dienes (Cook et al. in press), 22:1n-9, 20:20n-6, 20:3n-6 and 20:4n-6 (arachidonic).

DISCUSSION

All the manufactured diets were successful in promoting rapid gonad growth and created a more uniform gonad in terms of color and texture. The diets were palatable to the urchins, which adapted well to feeding on the pellets. Because the artificial diets did not differ greatly in composition, it is unsurprising their consumption rates were the same. However, it was anticipated that their consumption rate would have differed from that of the reference group fed *L. saccharina*, which had a higher fiber content. Bedford and Moore (1985) showed that adult *P. miliaris* reduced their gut retention times to compensate for less digestible materials. In the current experiment, the consumption rate data were collected from replicate groups of urchins; ingestion and assimilation could be re-examined for individual urchins fed artificial and reference diets.

Various artificial diets were able to promote somatic growth in other adult sea urchin species (Nestler and Harris 1994, Fernandez and Bourdouresque 1998). Protein is a constituent of the organic material (intrastereomic matrix) of the echinoderm body wall (Dubois and Chen 1989), and its incorporation in the diet has been proved beneficial for the somatic growth of other sea urchin species (Fernandez 1997, McBride et al. 1998). Adult *P. miliaris* (test diameter 15 mm) have also experienced increased somatic growth after the administration of a commercial salmon feed and for a

TABLE 2.

Gonad live weight (g) of sea urchins fed the artificial diets and *Laminaria saccharina* over the experimental period.

	Day 1 (n = 8)	Day 39 (n = 6–8)	Day 70 (n = 6–10)	Day 97 (n = 25–70)
Diet 1	0.26 (0.154)	0.34^{a} (0.12)	1.09^{a} (0.29)	1.67^{a} (0.71)
Diet 2	0.26 (0.154)	0.45^{a} (0.11)	1.67^{b} (0.24)	1.95^{a} (0.6)
Diet 3	0.26 (0.154)	0.36^{a} (0.12)	1.83^{b} (0.57)	1.71^{a} (0.56)
Reference	0.26 (0.154)	0.06^{b} (0.026)	0.26^{c} (0.13)	0.22^{b} (0.09)

Standard Deviation in parenthesis. Numbers in the same column and with the same superscript are not significantly different ($P < 0.05$).

TABLE 3.

Comparative gonad biochemistry of urchins fed experimental Diets 1–3, Day 97.

	Diet 1	Diet 2	Diet 3	Reference
Dry matter	27.30^{a,b} (1.54)	26.07^{b} (1.74)	26.736^{a,b} (0.72)	28.59^{a} (1.22)
Crude protein	42.46^{a} (0.77)	42.40^{a} (1.15)	41.51^{a} (1.98)	33.11^{a} (0.32)
Crude lipid	25.20^{b} (1.11)	15.05^{c} (5.84)	23.18^{b} (4.68)	42.65^{a} (1.56)
Glycogen	27.56^{a} (3.23)	22.62^{b} (6.58)	26.53^{a} (13.97)	15.15^{b} (3.42)
Ash	4.02^{b} (0.08)	5.01^{b} (1.85)	5.16^{b} (0.65)	8.33^{a} (1.55)
Energy kJ/g^{-1}	12.71^{a,b} (1.43)	14.71^{a,b} (3.28)	17.41^{a} (7.29)	11.99^{b} (2.35)

Values are expressed as percentage of the sample on a dry matter basis. Standard deviation in parenthesis (n = 3). Numbers in the same row and with the same superscript are not significantly different ($P < 0.05$). Statistics generated with arcsine transformed data.

period of 6 months (Cook et al. 1998). The artificial diets in this experiment did not promote measurable somatic growth; however, the trial was of relatively short duration for recording test growth. Calcium carbonate represents, roughly, 48% of the total shell volume of echinoderms (Weber 1969, Kaneko et al. 1982) and eventually accounts for a considerable portion of the inorganic carbon of the shell. Although the artificial diets used in this experiment had much lower ash levels (7–8% approximately, Table 1) than *L. saccharina* (28% of its dry weight; Black 1950), the urchins fed artificial diets maintained test ash values as high as those of the reference group, indicating that a high ash content in an artificial diet may not be essential for maintaining inorganic test content.

TABLE 4.

Fatty acid profiles of gonads and diets

	Gonads		Diets	
	Mean	SD	Mean	SD
14:0	3.3	0.9	0.3	0.3
15:0	0.2	0.2	0.0	0.0
16:0	11.3	1.3	11.3	0.7
16:1n-7	1.7	0.6	2.0	0.5
16:1*	0.4	0.6	0.0	0.0
18:0	2.5	0.4	4.0	0.5
18:1n9	5.6	0.9	22.4	0.8
18:1n-7	1.3	0.4	1.0	0.2
18:2n-6	24.2	2.7	52.0	2.3
18:3n-3	0.0	0.0	5.7	0.5
18:4n-3	0.0	0.0	0.0	0.1
20:1*	5.3	0.5	0.4	0.5
20:0	2.6	0.4	0.2	0.0
20:2*	8.2	0.6	0.0	0.0
20:2n-6	7.2	1.5	0.1	0.1
20:3n-6	2.9	0.6	0.0	0.0
20:4n-6	12.2	1.5	0.0	0.0
20:3n-3	0.2	0.3	0.0	0.0
20:4n-3	0.1	0.1	0.0	0.0
20:5n-3	1.7	0.7	0.4	0.2
22:1n-9	3.3	0.4	0.0	0.0
22:5n-3	0.0	0.0	0.0	0.0
22:6n-3	0.4	0.4	0.0	0.0
U/K	5.4	2.5	0.2	0.5
Totals	100.0		100.0	

Methyl esters as % of total methyl esters on a wet basis.
U/K = unknown peaks.
* = Double bond position not determined.

The higher mortality recorded for the reference group could possibly be attributed to the seasonal variation of the biochemical composition of the kelp (Black 1950) resulting in a deficient diet incapable of meeting the nutritional requirements of the urchin at this life stage. Similarly, Fong and Mann (1980) suggested that the amino acid profile of the *L. longicruris* was of low nutritional value for *Strongylocentrotus droebachiensis* (nutritional value assessed as percentage of each essential amino acid found in *L. longicruris* compared to the respective one found in egg albumin (Cowey and Sargent 1972).

The rapid gonad growth seen in the urchins fed the artificial diets is a positive attribute for a potential aquaculture species. The increase in GI from a mean of 2 to 17% over approximately 14 weeks compares favorably with the increase in GI found in urchins maintained in polyculture with Atlantic salmon, and accessing salmon feed, over a 16-wk period [GI increased from 2.56% (1.38 SD, n = 20) to 18.95 (2.39 SD, n = 20); data converted from that of Kelly et al. 1998b]. It is not known, however, if the GI would have continued to increase over time, as found for *P. miliaris* fed exclusively on salmon feed (Cook et al.1998). Because the manufactured sea urchin diets were composed of 36–37% crude protein and 5–9% crude lipid, they seem to be equally or even more cost effective, in terms of promoting gonad growth, to the salmon feed containing 40–45% crude protein and 20–30% crude lipid.

Although very different to natural dietary lipid sources, the mainly vegetable oil lipids in the diets seemed to allow good gonad growth in this species. There was no relationship between dietary lipid content and gonadal lipid storage, the level of which, therefore, must have been controlled by some other dietary or metabolic factor. The dietary lipid was provided by vegetable-derived oils rich in 18:1 and 18:2, comprising about 75% of the total dietary lipid. Using such starting materials with a low level of polyunsaturation, the urchins were capable of accumulating a range of 20 carbon fatty acids (20:2*, 20:2n-6, 20:3n-6 and 20:4-6) as well as some 22:1n-9, which must, therefore, be regarded as nonessential fatty acids for this species. The urchins also had small amounts (1.7%) of 20:5n-3 in their gonads, but this may have been accumulated directly from the diet, which also contained small amounts (0.4%). In addition, small amounts (0.4%) of 22:6n-3 were also present in the gonads despite its absence in the diets. This may have originated from dietary sources before the start of the experiment, because this species lacks the enzyme systems for desaturation/elongation necessary for synthesis of this fatty acid (Bell et al. in press). Because gonad growth was dramatic over the course of the experiment, the lipid profile of the gonads presumably largely results from the diets they were fed during the trial. Cook

et al. (in press) found that urchins held in aquaria and fed primarily on salmon feed had high levels of 22:6n-3, probably accumulated directly from the high levels in their diet. Further experimentation using labeled fatty acids will better clarify the biosynthetic ability of *P. miliaris* at this high level of polyunsaturation.

High levels of good quality fishmeal and fish oil rich in PUFAs constitute the basis for salmon diets, characterized by approximately 40–45% crude protein, 20–30% crude lipid and a high manufacturing cost. The satisfactory gonad growth of sea urchins fed diets composed of inexpensive vegetable oils, cereal grains, and agricultural by-products, advocates for the use of such diets in sea urchin culture. More experimental work to fully meet the sea urchins' nutritional requirements using cost-effective, low PUFA diets is needed.

Although the gonad color produced by the artificial diets was not the color the marketplace prefers (often described as pumpkin), the colors were bright and clear tones, and, therefore, not unattractive (except for those of the reference that were dark and brown). The noted improvement of the roe color in most of the experimental urchins calls for further research into the incorporation and expression of dietary pigments.

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SPATIO–TEMPORAL DISTRIBUTION OF *PROROCENTRUM LIMA* IN COASTAL WATERS OF THE GULF OF MAINE: A TWO-YEAR SURVEY

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ABSTRACT The dinoflagellate *Prorocentrum lima* (Ehrenberg) Dodge was found at several sites along the coast of Maine in 1998 and 1999, some in areas where shellfish are harvested commercially. Identity was confirmed by scanning electron microscopy (SEM). Although this dinoflagellate is known to produce toxins (okadaic acid and derivative compounds), incidence of diarrhetic shellfish poisoning in coastal Maine is not well understood, despite confirmed toxicity events in the early 1990s in Nova Scotia, Canada in adjoining waters to the north. Some samples containing the dinoflagellate came from wild mussel populations collected at low tide, while others originated from aquaculture sites. Many of the cells were isolated from water samples and net tows and on a few occasions were associated with filamentous macroalgae. *Prorocentrum lima* appears to be relatively rare in Maine coastal waters, but its widespread distribution over several months warrants increased monitoring to allay public health concerns.

KEY WORDS: *Prorocentrum lima*, diarrhetic shellfish poisoning, DSP, Gulf of Maine

INTRODUCTION

Diarrhetic shellfish poisoning (DSP) is a severe gastrointestinal illness in humans resulting from the ingestion of shellfish containing DSP toxins (okadaic acid and derivatives). These toxins accumulate in shellfish that have been feeding on dinoflagellates of the genus *Prorocentrum* Ehrenberg or *Dinophysis* Ehrenberg. In coastal northeast USA, knowledge of DSP incidence is fragmentary and until recently, no DSP monitoring has been judged necessary. However, three events of the past 10 years challenged this complacent perspective: (1) in the late 1980s, several shipments of the oyster *Ostrea edulis* Linnaeus, originating from Maine and sent oversea, tested positive for DSP and were refused at a great economic loss to shellfish farmers (Shumway 1990). The source of DSP toxins was not determined and remains controversial. (2) In 1990, the first confirmed DSP event in North America involved cultured mussels on the Atlantic coast of Nova Scotia, Canada, in waters contiguous with the Gulf of Maine (Quilliam et al. 1993). The DSP toxicity was linked to populations of *P. lima* (Jackson et al. 1993, Lawrence et al. 1998). (3) *Prorocentrum lima*, a known toxin producer, was first observed in the Gulf of Maine in 1994 in an offshore plankton net sample collected in the Great South Channel, west of Georges Bank (Maranda et al. 1999), but, until then, had not been reported from coastal locations.

These events prompted us to investigate the extent, in space, time and abundance, of the presence of *P. lima* in the coastal waters of the Gulf of Maine. Recently, Morton et al. (1999) detected low levels of okadaic acid-like activity in blue mussels *Mytilus edulis* Linnaeus and reported on the presence of *P. lima* in one area along the coast of Maine.

MATERIAL AND METHODS

Wild blue mussels *M. edulis* and associated sediments were collected at low tide once a month in March, April, May, and October, and twice a month in June, July, and August, at ten monitoring stations along the coast of Maine in 1998 and 1999 (Fig. 1). This sampling strategy was driven by the economic impact *P. lima* can potentially have on the shellfish industry. Epibiota and associated sediments were rinsed off mussels with 0.45-μm filtered seawater and the 10- to 90-μm size fraction was examined in a Sedgwick–Rafter chamber by bright field microscopy using a 10X or 20X objective. Cells resembling *P. lima* were isolated with a stretched pipet for observation at higher magnification or for preparation for scanning electron microscopy (SEM). Whenever possible, samples were observed fresh, otherwise they were pre-

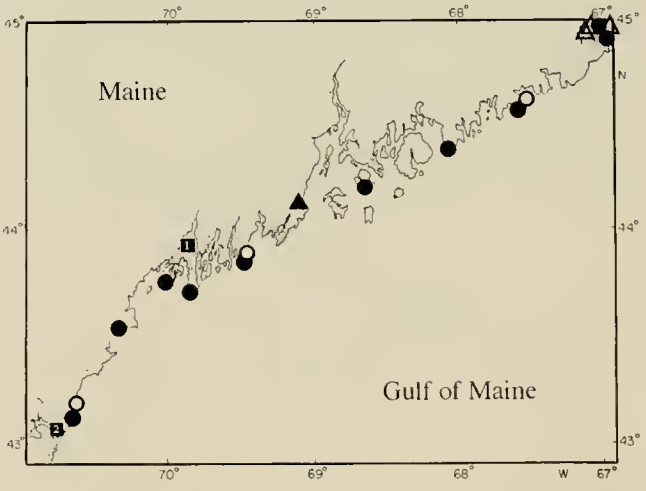

Figure 1. Coast of Maine. Location of ten stations (●) sampled for epibionts on mussels, three subtidal (○) and three intertidal (△) stations sampled for epibionts on macroalgae, two commercial shellfish sites (■) and Glen Cove, also known locally as Clam Cove, a mid-coast station (▲).

served in formaldehyde:acetic acid (1% final concentration) and examined within 6 mo of collection. Sample size (mussel number and size) varied with stations: total weight of material ranged between 0.2 and 1.7 kg, with an average of 45% in sediments and epibionts.

Whole water samples and/or plankton samples (>10 μm) were collected at additional stations on an irregular basis: a shellfish nursery site (fall 1998 and summer 1999), a shellfish depuration site (summer 1999), and Glen Cove, a shallow mid-coast site with abundant wild mussels (summer and fall 1999) (Fig. 1). For 3 mo during the summer of 1998, whole specimens of filamentous macroalgae and associated epibiota were collected underwater twice a month at three subtidal stations by a scuba diver (Fig. 1); phaephophytes and rhodophytes were harvested down to 10-m depth. In the fall of 1999, macroalgae and associated epibiota were collected at three intertidal sites in the northeast section of coastal Maine (Fig. 1). The epibiota was later shaken off seaweed specimens; the samples were settled and concentrated 20 to 50 times prior to observation in a Sedgwick–Rafter chamber.

Criteria used for the identification of *P. lima* were those defined by Faust (1991).

RESULTS

1998

From the epibionts collected on mussels (99 samples), cells of *P. lima* were detected in a tide pool at Head Beach, 43°43′N 69°51′W, in October (Fig. 2 and Fig. 3A and B). During that same month, one of us (M. D. K.) collected *P. lima* at a shellfish nursery site, 43°55′N 69°52′W (filled square #1, Fig. 2) and established a mixed culture. *P. lima* was reisolated in clonal cultures several months later (Fig. 4) and deposited at the Provasoli–Guillard Center for Culture of Marine Phytoplankton (CCMP1966). Size and pore pattern of the cells falls within published values for the species (Table 1).

The epibiota of filamentous macroalgae from subtidal locations did not harbor any *P. lima* cells.

1999

In August, September and October, cells of *P. lima* were identified by SEM in whole water and plankton samples, and with

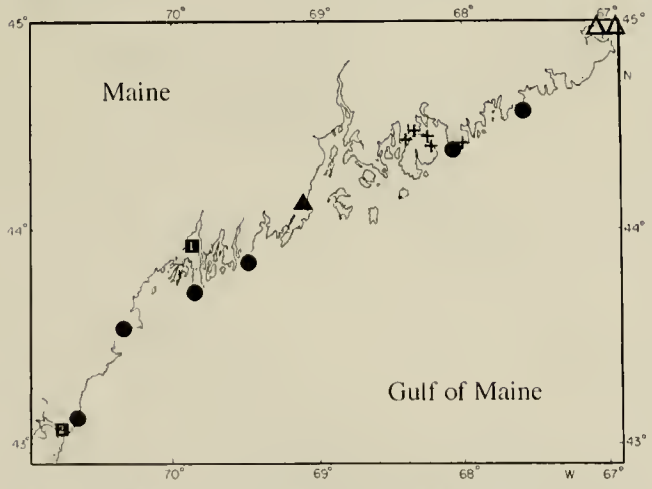

Figure 2. Location of stations where *P. lima* was found in 1998 and in 1999. Symbols as in Figure 1, except for the sites (crosses) reported by Morton et al. (1999 and personal communication).

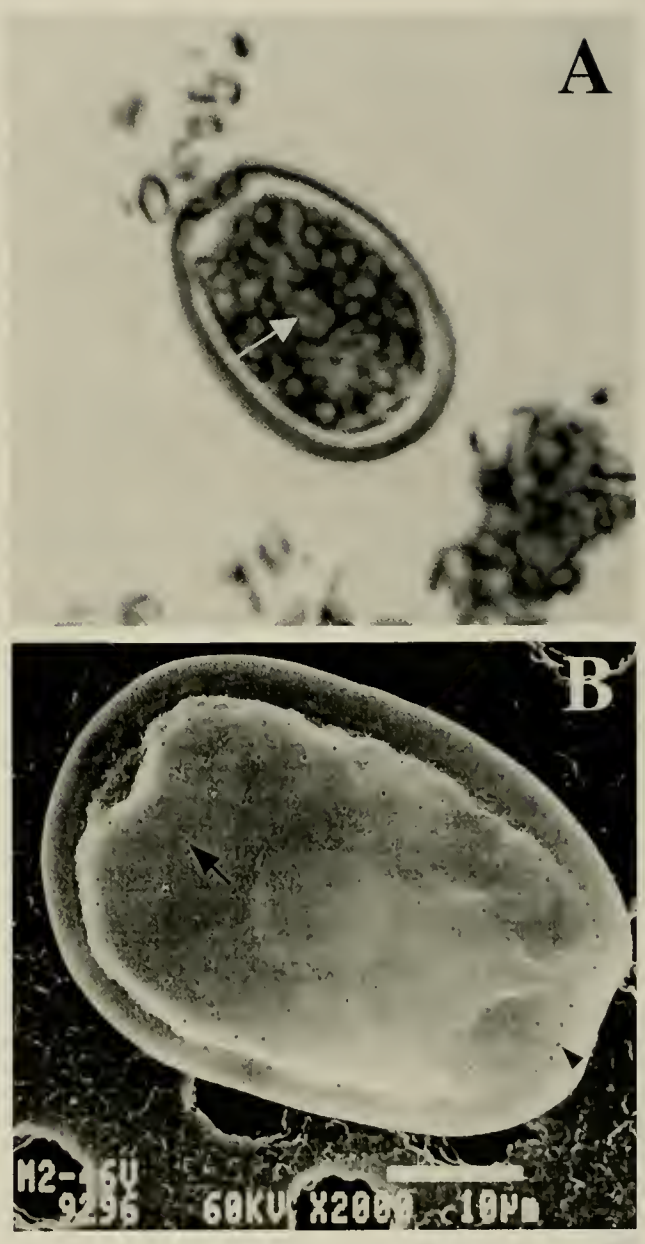

Figure 3. *Prorocentrum lima* from a tide pool near Head Beach, ME, October 1998. (A) Bright field photomicrograph of one cell within detritus as seen in a Sedgwick–Rafter counting chamber. The arrow points to the central pyrenoid. Note the depression in the apical area on the right thecal plate. (B) SEM micrograph of the left thecal plate of a second cell isolated from Head Beach. Note the row of marginal pores (arrowhead) and the pattern of the valve pores (arrow).

filamentous macroalgae from Glen Cove, 44°08′N 69°06′W and, in August and September, were found at the same shellfish nursery site sampled in 1998 (Fig. 2).

Cells of *P. lima* were observed by light microscopy in 5 of 105 samples of mussel epibionts analysed. They came from five different stations spanning the whole coast of Maine and were collected between June and September. In September, cells were found in water samples from a shellfish depuration site (filled square #2) and within the epibiota of two macroalgal collections (Fig. 2). Shape and size of cells fall within the reported range for *P. lima*.

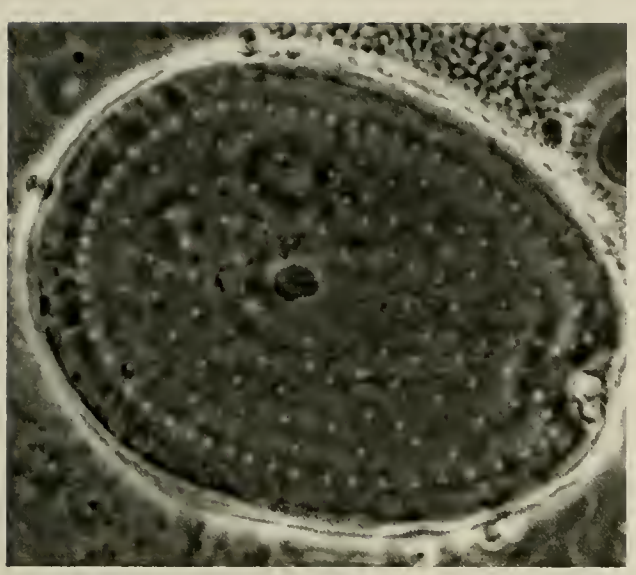

Figure 4. *Prorocentrum lima* from cultured material originating from the shellfish nursery site #1. Collapsed right thecal plate, treated with chloral hydrate. Oil immersion phase contrast.

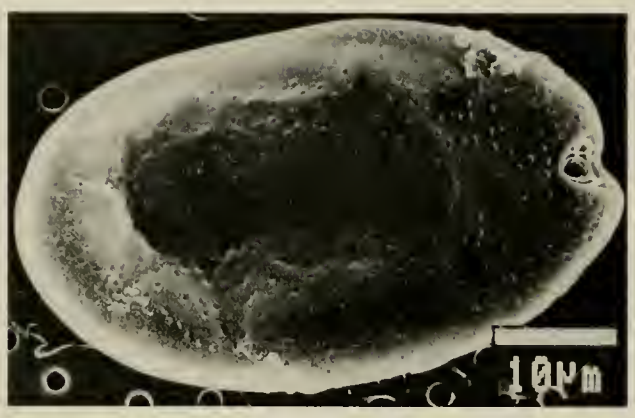

Figure 5. *Prorocentrum cassubicum* from Mosquito Harbor (44°23′N 68°04′W), October 1997. SEM micrographs reveal a high number of marginal and valve pores (ca. 120 and 218, respectively). The size (51 × 30 µm, L/W ratio: 1.70) and shape of the cell come close to that of *P. lima*.

DISCUSSION

Prorocentrum lima is present in coastal waters of the Gulf of Maine. The SEM-based identification at three sites is unequivocal and included size, shape and micro-morphological characters of cells associated with mussels and in water samples. The pattern and number of marginal and valve pores, along with the apical depression on the right theca, constitute the most compelling evidence of the identity of the cells. *Prorocentrum lima* was also detected at several other sites along the coast of Maine, although micro-morphological characters could not be resolved in all cases by light microscopy. We recommend that cells be identified with attention to micromorphology, especially at low magnification, as non-toxic *Prorocentrum* species such as *cassubicum* (Woloszynska) Dodge (Fig. 5) can easily be mistaken for *lima* especially in samples loaded with sediments and detritus.

It is surprising that, despite many decades of phytoplankton studies in the Gulf of Maine and surrounding coastal waters, no reports of *P. lima* can be found prior to 1994 (Maranda et al. 1999). Similarly, *P. lima* was not recorded from eastern Canadian waters prior to the 1990 DSP incident. Whether this predominantly benthic/epiphytic toxin producer was a previously "hidden" or not appropriately sampled native member of local populations or whether a successful genotype has been recently introduced in the northwest Atlantic cannot be resolved at this time. Nevertheless, the presence of *P. lima* signals the potential for DSP incidence given that, to date, all cultured clones identified as *P. lima*, regardless of their origin, produce OA and/or some derivatives (McLachlan et al. 1997, Morton et al. 1999).

In Canadian waters, *P. lima* grows in association with filamentous macroalgae fouling mussels suspended in long-line cultures (Lawrence et al. 1998). Shellfish may thus become toxic after feeding on the epiphytic *P. lima* when it becomes available as a food item following turbulence in the water column. In Maine, mussel growers use predominantly on-bottom cultivation methods, less susceptible to fouling macroalgae, while wild mussels still constitute an important portion of annual landings. However renewed interest in and conversion to suspension methods are gaining momentum: this could lead to increased exposure of mussels to fouling macroalgae and associated flora, including the toxic *P. lima*. This specific association between *P. lima* and filamentous macroalgae is documented in tropical/subtropical habitats as well (Tindall and Morton 1998) and implies that our spatio–temporal survey of mussel epibionts may underestimate the dinoflagellate population. Appropriate quantitative sampling of plankton and epiphytic populations would thus be needed to undersatnd the ecology of *P. lima* in Maine coastal waters.

Although our survey suggests that *P. lima* is relatively rare at most stations, the widespread distribution of the dinoflagellate, its recurrence 2 y in a row and over several months, and presence close to mussels and in the plankton warrant increased monitoring to address public health concerns, especially in light of coming shifts in cultivation methods.

TABLE I.

Comparison of *Prorocentrum lima* from the coast of Maine with some published micro-morphological features (length and width in µm).

Reference/Soorce	Length	Width	L/W Ratio	Marginal Pores	Valve Pores
Dodge 1982	32–50	20–28	—	—	—
Faust 1991	31–47	22–40	1.18–1.52	55–72	58–86
Marr et al. 1992	46	32	1.44	ca. 55	ca. 70
Morton and Tindall 1995	38–41	32–36	1.18–1.34	—	—
Head Beach (two cells)	43, 48	28, 35	1.39, 1.54	73, 79	76, 91
Glen Cove	46–50	32–33	1.44–1.51	56–60	ca. 60
Shellfish nursery site	41–50	26–36	1.49	56–60	ca. 70

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ENVIRONMENTAL CONDITIONS ASSOCIATED WITH DOMOIC ACID IN RAZOR CLAMS ON THE WASHINGTON COAST

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ABSTRACT In October 1998, record levels of the neurotoxin domoic acid (DA) were detected in razor clams (*Siliqua patula*, Dixon) resulting in the closure of shellfish harvesting areas along the Washington coast. This toxin was detected in seawater samples collected at Kalaloch Beach and Second Beach on the central Washington coast using a receptor binding assay and liquid chromatography-tandem mass spectroscopy. Domoic acid levels ranging from 0–2700 ng/L were measured in seawater samples containing from 70–100% *Pseudo-nitzschia pseudodelicatissima* (Hasle) Hasle at concentrations of $1.0–15 \times 10^6$ cells/L, resulting in maximum levels of cellular toxin of approximately 500 fg/cell. A cultured isolate of this species collected from Kalaloch Beach also produced DA, as determined by the receptor binding assay, during late exponential and stationary stages of growth. The toxic *P. pseudodelicatissima* bloom in the late summer and autumn of 1998 occurred 2–3 weeks after strong coastal upwelling during a period of anomalously low rainfall, typical in post-El Niño years. Higher toxin levels in seawater at Kalaloch Beach compared to Second Beach were attributed to the periodic nature of upwelling at Kalaloch Beach, demonstrated by a 175-fold increase in nitrate in seawater coincident with a 5 °C decrease in sea surface temperature on September 1. The upwelling event in September was followed by wind relaxation and reversal at the end of that month, resulting in the transport of toxic cells toward the coast where nutrients were already present to fuel the algal bloom. A pulse of nutrients, either from rainfall or upwelling, to coastal regions that have experienced several weeks of low nutrients, followed by wind relaxation or reversal events that transport cells to inshore regions, are suggested to be important factors in the initiation of the most toxic *Pseudo-nitzschia* species blooms on the Washington coast.

KEY WORDS: *Pseudo-nitzschia*, domoic acid, razor clams, upwelling

INTRODUCTION

The first domoic acid (DA) poisoning of humans was reported in 1987 in eastern Canada (Todd 1993). A series of collaborative studies demonstrated that the toxin was concentrated in mussels that had fed on a bloom of *Pseudo-nitzschia multiseries*, the first pennate diatom from which DA was isolated (Bates et al. 1989). In 1991, DA was implicated in the illness and death of brown pelicans (*Pelecanus occidentalis*) and Brandt's cormorants (*Phalacrocorax penicillatus*) in Monterey Bay, California (Work et al. 1993). Using laboratory isolates, Garrison et al. (1992) determined that *P. australis* was the DA-producing diatom responsible for the mortalities. Unlike the outbreak in eastern Canada, where mussels were the toxin vector (Wright et al. 1989), anchovies became toxic to seabirds after feeding on the diatom, *P. australis*, in Monterey Bay (Buck et al. 1992, Fritz et al. 1992), illustrating that DA has at least two means of entering the higher food web, through filter-feeding molluscan shellfish and suspension-feeding finfish.

The poisoning event in Monterey Bay resulted in the establishment of a DA monitoring program in the states of Washington and Oregon. In October 1991, about 1 month following the toxic bloom event in California, levels of DA above the regulatory limit of 20 μg/g shellfish tissue were found in the edible parts of razor clams (*Siliqua patula*) and Dungeness crabs (*Cancer magister*) collected on the Washington coast (Wekell et al. 1994). Consequently, beaches were temporarily closed to recreational and commercial shellfish harvesting (Horner and Postel 1993), resulting in a substantial loss of revenue ($15–20 million) to local fishing communities (Anderson 1995). The *Pseudo-nitzschia* species responsible for the DA poisoning event was not determined, because there were no phytoplankton samples collected on the Washington coast immediately before the measurement of toxin in razor clams (Horner et al. 1993). Several species that had been determined to be toxic in some, but not all, geographical regions (i.e., *P. multiseries*, *P. australis*, *P. pungens*, and *P. pseudodelicatissima*) were found in Washington coastal waters (Horner et al. 1997). Among those species, *P. australis* was thought to be responsible for the presence of DA, because it was identified in samples collected off Grays Harbor, Washington, several months after the initial event (Horner and Postel 1993). Taylor and Horner (1994) suggested that the 1991 toxic bloom event off the Washington coast might have been part of a widespread bloom of *P. australis*. It was estimated that a bloom starting in California in September 1991 and carried up the coast by currents at speeds of nearly 20–40 cm/sec could have reached the Washington coast by late October to November (Horner et al. 1997).

More recently, in May and June of 1998, the first confirmed deaths of a marine mammal species attributable to DA poisoning were documented in sea lions along the central California coast. In this mortality event, sea lions fed on anchovies and sardines that

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had ingested toxic *P. australis* cells (Gulland et al. 1999, Lefebvre et al. 1999, Scholin et al. 2000). Because razor clam toxification on the Washington and Oregon coasts in 1991 was preceded by the death of seabirds in Monterey Bay earlier that summer, we suspected that the poisoning of sea lions by DA in 1998 was an early warning of impending toxicity on the Washington and Oregon coasts.

Because concentrations of *Pseudo-nitzschia* spp. increase and subside rapidly in Washington coastal waters (R. Horner, pers. comm.), a more rigorous and frequent monitoring effort was implemented by sampling at two beaches in order to investigate the relationships among *Pseudo-nitzschia* spp. cells, DA levels in seawater and shellfish, nutrients, and a variety of environmental variables. This paper describes weekly sampling of the surf zone, which enables us to describe the chemical, biological, and physical processes preceding and during the razor clam toxification event that occurred in the late summer and early autumn of 1998.

METHODS

Sample Collection

Seawater was sampled on a weekly basis from the surf zone of two accessible beaches on the Pacific coast of Washington State: Second Beach, near La Push, and Kalaloch Beach, approximately 65 km south of La Push (Fig. 1). Seawater samples were collected using a bucket and preserved for phytoplankton species identification and enumeration, as well as DA, chlorophyll *a*, and nutrient analyses as described below.

Nutrient and Chlorophyll a Analyses

Seawater was filtered through a 25-mm Whatman #1 filter and collected in a 60-mL polyethylene bottle, frozen, and later analyzed for nutrient concentrations using standard autoanalyzer methods (Whitledge et al. 1981). Aliquots (50 mL) of seawater samples were filtered through Whatman GF/F filters for chlorophyll *a* detection. Filters were stored at −20 °C until analysis by extraction with 10 mL 90% acetone overnight at 4 °C in the dark. Extracts were analyzed using the standard fluorometric method (Welschmeyer 1994) using a Turner Designs (TD-700) fluorometer with narrow bandpass filters.

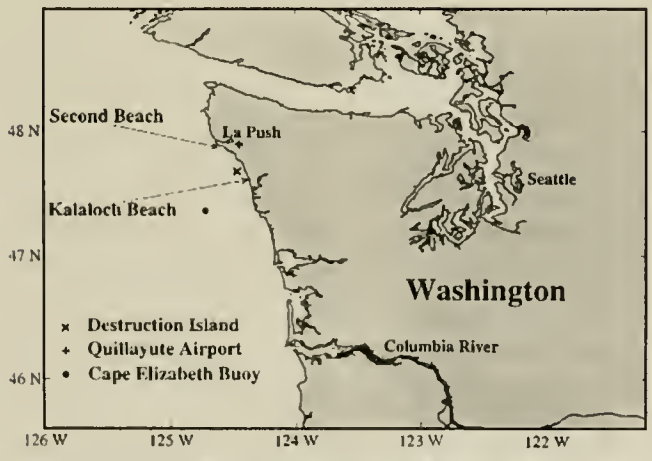

Figure. 1. Sampling sites on the central Washington coast and locations of coastal environmental monitoring stations.

DA Analysis in Phytoplankton

Cellular DA was measured by filtering 2 liters of seawater through a 47-mm 0.45-μm (Millipore HA) filter. Depending on the density of the material in the sample, up to three filters were used. Filters were folded in half with forceps, wrapped in aluminum foil, and frozen until analysis using the method described by Van Dolah et al. (1997). A glutamate decarboxylase digestion step was used before analysis to remove endogenous glutamate in all samples. Binding experiments were initiated by incubation of 100 μL of cloned GluR6 membrane preparation (Taverna and Hampson 1994) with 50 μL of a 5 nM solution of [^{3}H] kainic acid and 50 μL of standard or sample in a 13 × 10 mm glass test tube. Samples were vortexed briefly, incubated at 4 °C for 1 h, poured over Whatman GF/C filters (25-mm diameter), and rinsed twice with 5 mL of 50 mM Tris-citrate buffer (pH 7.4). Filters were placed in scintillation vials, soaked overnight in 10-mL scintillation fluid, and radioactivity was measured using scintillation spectroscopy. For quantification of DA in selected seawater samples, liquid chromatography-tandem mass spectroscopy (LC-MS/MS) was performed according to standard protocols (Scholin et al. 2000).

Cell Counts

Phytoplankton cells were counted by first pouring 100 mL of seawater into a graduated cylinder, then fixing with formaldehyde to a final concentration of approximately 1 % and settling for 24–72 hours. Ninety mL of water were carefully drawn off with a pipette and the settled material was resuspended. A 0.1 mL subsample was loaded into a Palmer–Maloney slide, and a minimum of 100 individual algal cells were counted at 100X magnification using light microscopy. Phytoplankton cells were identified to the lowest possible taxon and to species when possible. The percentage contribution of the *Pseudo-nitzschia* species for the whole phytoplankton assemblage, viewed at 100X, was calculated.

Scanning Electron Microscopy

In samples where *Pseudo-nitzschia* spp. were numerous, aliquots were prepared using a modified $KMnO_4$/HCl oxidation method (Miller and Scholin 1998). Filter membranes with processed samples were bonded to aluminum stubs, air-dried, coated with gold-palladium, and examined with an AMRAY 1000 SEM.

Growth Study

Pseudo-nitzschia pseudodelicatissima, clone NWFSC-047, isolated from a plankton sample collected at Kalaloch Beach, Washington on July 31, 1999, was grown in batch culture. The isolate used in the growth study was not axenic, but sterile technique was used throughout the experiment. Cells were maintained in f/2 medium (Guillard and Ryther 1962) made with filtered natural seawater. An inoculum of exponentially growing cells was used. Duplicate cultures (200 mL each) were grown in 1-L borosilicate culture flasks at 13 °C. Irradiance was provided by a bank of Cool-White fluorescent lamps (15 W). Cells were exposed to a 12:12 h light:dark cycle at a light intensity of 80 μE m^{-2} s^{-1}. Every 3–4 days during the study, 20-mL aliquots from each flask were filtered onto Millipore HA, 0.45-μm, 25-mm diameter filters using vacuum (< 15 psi). Filters were stored and analyzed for DA by receptor binding assay as described above, and a 0.1-mL aliquot of culture was collected for cell enumeration.

Environmental Data

Wind speed and direction were obtained from the National Data Buoy Center's weather station on Destruction Island, Washington (NDBC #DESW1, 47.68 °N, 124.49 °W), which is about 7 km northwest of Kalaloch Beach. Rainfall data were obtained from the Quillayute airport, near La Push, Washington and from the Kalaloch Ranger Station in Kalaloch, Washington. Water temperature at beach sampling sites was measured with a thermometer. Sea surface temperatures were obtained from the Cape Elizabeth buoy (NDBC #46041) at 47.4 °N, 124.5 °W. The locations of coastal monitoring stations are shown in Figure 1.

Domoic Acid Analysis in Shellfish

Razor clams were collected at Kalaloch Beach for DA analysis. Concentrations of DA were determined utilizing a methanol/water extraction and analysis by high-performance liquid chromatography (HPLC) (Hatfield et al. 1994). Additional razor clam toxin concentration data were obtained from samples collected by Quileute Natural Resources staff and analyzed by the Washington Department of Health (WDOH). Only the edible parts of the clams were analyzed for DA (i.e., viscera were not analyzed).

RESULTS

Pseudo-nitzschia Cell Numbers and Domoic Acid Levels

As *Pseudo-nitzschia* spp. cell counts increased, there was a corresponding increase in the level of DA activity detected in seawater at Kalaloch Beach (Fig. 2a). Both cell counts and DA activity in seawater reached a maximum on September 22 at 17.1 × 10^6 total *Pseudo-nitzschia* spp. cells/L and 2,700 ng DA/L seawater. A smaller peak of DA activity on August 25 also corresponded with elevated *Pseudo-nitzschia* spp. cell counts. The highest recorded level of DA detected in razor clams (295 μg/g) occurred within 18 days after the maximum *Pseudo-nitzschia* spp. cell number and level of seawater toxicity were measured.

At Second Beach (Fig. 2b), the highest number of *Pseudo-nitzschia* spp. cells was about three times lower than that at Kalaloch Beach, reaching a maximum of 5.9 × 10^6 cells/L on September 23; whereas, the level of DA activity detected in seawater reached a maximum of 350 ng/L on October 6. There were also smaller peaks in *Pseudo-nitzschia* spp. cell counts on August 12 and 26 associated with increased DA activity in seawater. An increase in *Pseudo-nitzschia* spp. cells on September 9 did not correspond to elevated DA in seawater; however, toxin levels increased by September 13, corresponding to levels of 2.2 × 10^6 total *Pseudo-nitzschia* cells/L. Razor clams were not collected from Second Beach for measurement of DA.

Phytoplankton Assemblage Observations

Pseudo-nitzschia pseudodelicatissima (Fig. 3) was the only known DA-producer present in significant numbers within the phytoplankton assemblage (Table 1). Most of the other *Pseudo-nitzschia* were *P.* cf. *heimii* (up to 90% of the total *Pseudo-nitzschia* on August 25 at Kalaloch Beach) plus relatively small amounts of *P. pungens* (less than 3% on dates when SEM was done) and *P. delicatissima* (8% on September 1 at Kalaloch Beach, the only sample in which this species was observed).

Table 1 shows *Pseudo-nitzschia* spp. as a percentage of the whole phytoplankton assemblage and *P. pseudodelicatissima* as a percentage of total *Pseudo-nitzschia* spp. at Kalaloch and Second Beaches. At both sampling sites, *Pseudo-nitzschia* spp., as a percent of total phytoplankton species, increased until the end of

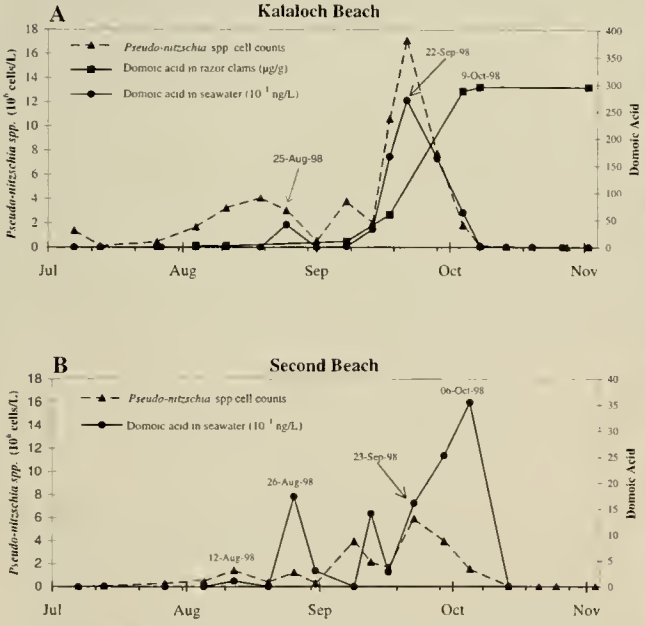

Figure. 2. Weekly *Pseudo-nitzschia* spp. cell counts and domoic acid in seawater from July through October 1998, at Kalaloch Beach (A) and Second Beach (B). Domoic acid levels in razor clams are shown for Kalaloch Beach.

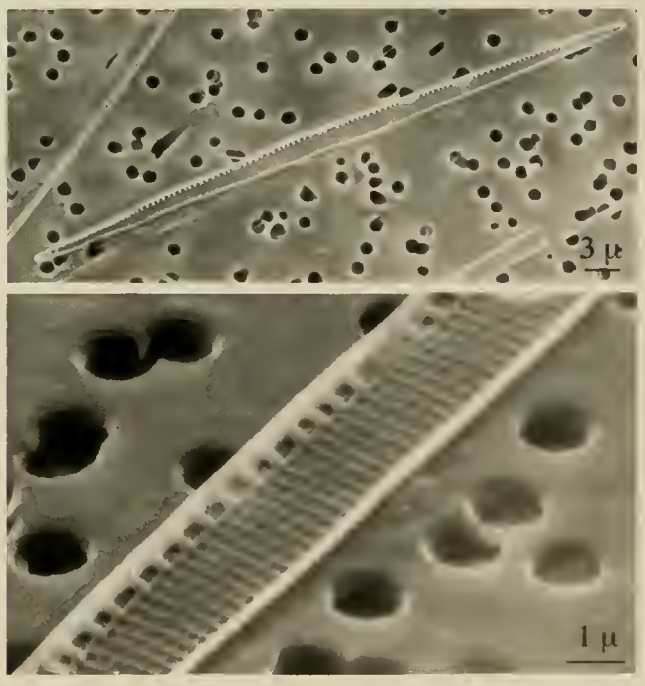

Figure. 3. Scanning electron micrographs of a field isolate of *P. pseudodelicatissima* from Kalaloch Beach. (top) a whole valve. (bottom); higher magnification showing 1 row of square poroids between interstriae. Scale bars indicate size.

TABLE 1.

Pseudo-nitzschia spp. abundance and domoic acid levels on the central Washington coast in 1998.

Kalaloch						
Date	*Pseudo-nitzchia* spp. (10^6 cells/L)	*Pseudo-nitzchia* spp. abundance[1]	Domoic Acid (ng/L)	Domoic Acid (fg/cell)[2]	Domoic Acid by LC-MS/MS	*P. pseudodelicatissima* abundance[3]
7 July	1.4	37	0			
13 July	0.1	30	0			
26 July	0.5	34	0		–	
4 Aug	1.7	48	0			
11 Aug	3.2	30	0			
19 Aug	4.1	59	0			40
25 Aug	3.0	64	410			
1 Sept	0.6	41	0			70
8 Sept	3.8	83	10			90
14 Sept	2.0	93	330			
18 Sept	10.6	97	1700	170	+	90
22 Sept	17.1	99	2700	180	+	90
29 Sept	7.8	99	1600	210	+	100
5 Oct	1.8	92	640	500	+	70
9 Oct	0.1	41	10			20
15 Oct	0.0	15	0			
21 Oct	0.0	16	0			
29 Oct	0.0	4	no data			
3 Nov	0.0	3	0			

Second Beach						
Date	*Pseudo-nitzchia* spp. (10^6 cells/L)	*Pseudo-nitzchia* spp. abundance[1]	Domoic Acid (ng/L)	Domoic Acid (fg/cell)[2]	Domoic Acid by LC-MS/MS	*P. pseudodelicatissima* abundance[3]
7 July	0.0	18	0			
13 July	0.1	38	0			
27 July	0.3	54	0			
5 Aug	0.5	56	0			60
12 Aug	1.4	78	10			60
20 Aug	0.4	71	0			
26 Aug	1.2	88	170	160		90
31 Aug	0.3	84	30			60
9 Sept	3.9	95	0			
13 Sept	2.2	97	140	109		90
17 Sept	1.7	99	30			
23 Sept	5.9	99	160	40		70
30 Sept	3.9	100	250			
6 Oct	1.6	98	360	230	+	100
15 Oct	0.0	50	0			
22 Oct	0.0	3	no data			
26 Oct	0.0	26	no data			
4 Nov	0.0	6	no data			

[1] as a percentage of the phytoplankton assemblage viewable at 100X; [2]domoic acid per cell was only calculated where DA was > 100 ng/L, and *P. pseudodelicatissima* abundance was >70%; [3]estimated values as a percentage of total *Pseudo-nitzschia* spp.

September and early October, at which time the percentage declined. The increase was due to a bloom of *P. pseudodelicatissima*, which comprised the majority of all *Pseudo-nitzschia* observed. *Pseudo-nitzschia pseudodelicatissima* reached maximum numbers on September 22–23, corresponding to measurable levels of DA at both sites and the highest measured DA levels at Kalaloch Beach. After October 6, numbers of *Pseudo-nitzschia* spp. declined dramatically, as did toxin levels at both sites. The LC-MS/MS results confirm that DA was present on September 18, 22, 29, and October 5 at Kalaloch Beach and on October 6 at Second Beach.

Nutrients and Chlorophyll a

Figure 4 shows the concentrations of phosphate, silicate, and nitrate relative to *Pseudo-nitzschia* spp. cell counts at Kalaloch Beach and Second Beach (*Pseudo-nitzschia* spp. cell counts are duplicated from Fig. 2). On July 26, there was an increase in the levels of all three nutrients at Kalaloch Beach (Fig. 4a). Phosphate and silicate increased by a factor of approximately 4 compared to the previous sampling date, and nitrate increased by a factor of about 40. Approximately 3 weeks after the influx of nutrients, there was a subsequent rise in the number of *Pseudo-nitzschia* spp.

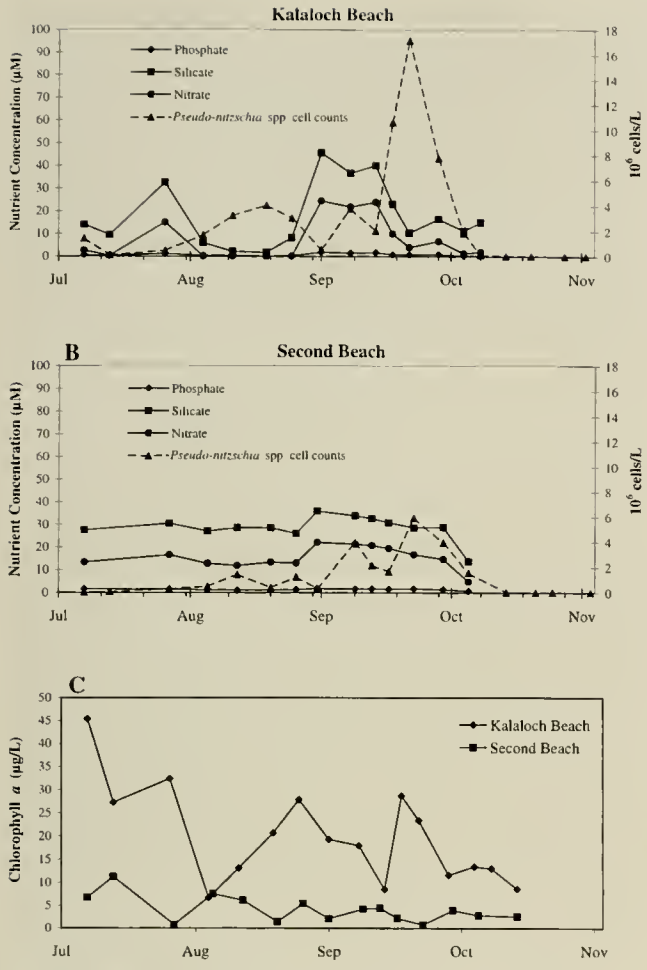

Figure. 4. Weekly concentrations of nitrate, silicate, phosphate from July through October 1998, at Kalaloch Beach (A) and Second Beach (B). *Pseudo-nitzschia* spp. cell counts are duplicated from Figure 2 for reference. Weekly chlorophyll *a* concentrations at Kalaloch Beach and Second Beach are shown (C).

cells that reached a maximum of 4×10^6 cells/L in August. There was another increase in nutrient levels on September 1; phosphate and silicate increased by a factor of 5 from the previous sampling date, and nitrate increased by a factor of 175. Until about September 14, nutrient levels remained relatively constant at Kalaloch Beach, with phosphate ranging from 1–2 µM, silicate 37–46 µM, and nitrate 22–24 µM, with the highest levels occurring on September 1. About 2–3 weeks following this second increase in nutrients, *Pseudo-nitzschia* spp. cell numbers increased from 0.5×10^6 cells/L on September 1 to 2.0×10^6 cells/L on September 13. Nutrient levels decreased substantially on September 18, corresponding to an increase in the number of *Pseudo-nitzschia* spp. to 10.6×10^6 cells/L. *Pseudo-nitzschia* spp. cell numbers reached their maximum of over 17×10^6 cells/L several days later on September 22, while nutrients continued to decline. After reaching this peak, *Pseudo-nitzschia* spp. cell counts rapidly decreased and remained low throughout the remainder of 1998. Chlorophyll *a* levels at Kalaloch Beach were routinely higher than those at Second Beach, except for the August 5 sampling date, when they were similar (Fig. 4c).

Second Beach showed more sustained high levels of nutrients compared to Kalaloch Beach (Fig. 4b). At Second Beach from July 7 to September 30, phosphate ranged from 1–2 µM, silicate from 26–36 µM, and nitrate from 12–22 µM. The concentrations of these nutrients peaked on August 31. After nutrient concentrations reached their maximum, there were two distinct peaks in *Pseudo-nitzschia* spp. cell numbers (4×10^6 cells/L on 20 August and 6×10^6 cells/L on September 23), which coincided with slightly decreased nutrient concentrations. On September 30, the number of *Pseudo-nitzschia* spp. cells began to decrease and on October 6 the levels of nutrients also began to decline. Chlorophyll *a* levels at Second Beach were less than 10 µg/L on all sampling dates (Fig. 4c).

Meteorological and Oceanographic Conditions

Monthly precipitation and sea surface temperatures (SST) are shown in Table 2 (see Fig. 1 and Methods for the locations of these monitoring sites). Average values for June through October for the period of record are compared to data for those months in 1998. Water temperatures for 1998 were similar to levels documented during the period of record (1987–1993). However, levels of precipitation for the summer of 1998 were lower than the average for the period of record (1948–1997), with September 1998 values almost an order of magnitude less than the average. There were also comparatively low amounts of rainfall at Kalaloch Beach during August and September of 1998 (B. Rhode pers. comm.).

Wind vectors are shown in Figure 5a. Data from the end of July through September showed periods of upwelling favorable winds (vectors pointing toward the southeast), with the most sustained period of strong upwelling in late August through early September. Periods of wind relaxation and mild reversal were common

TABLE 2.

Monthly mean sea surface temperature and precipitation on the Washington coast.

	Period of Record				
	June	**July**	**August**	**September**	**October**
Precipitation (cm) 1948–1998	8.0	6.6	5.7	12.2	26.4
Sea surface temperature (°C) 1987–1993	12.9	13.5	13.8	13.2	12.3
	1998				
	June	**July**	**August**	**September**	**October**
Precipitation (cm)	3.2	5.3	0.4	1.3	20.8
Sea surface temperature (°C)	12.6	13.8	14.0	12.4	12.6

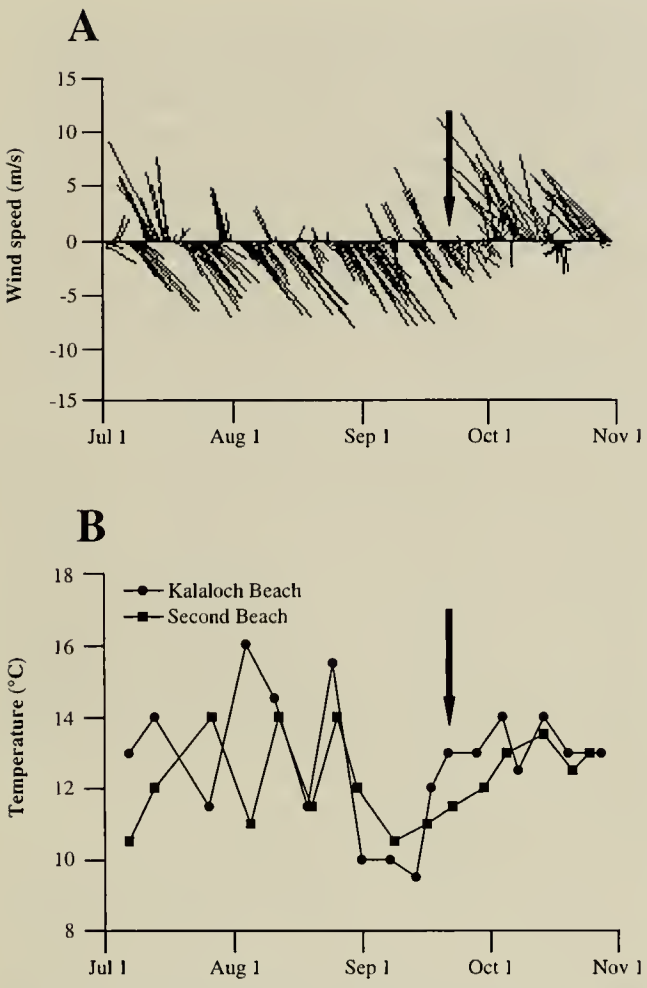

Figure. 5. A. Vector time series of daily average winds from June through October 1998. Length of lines refer to speed, angle of vector refers to direction. B. Weekly sea surface temperature at Kalaloch Beach and Second Beach from July through October 1998. Solid arrows indicate the date when the maximum numbers of *Pseudo-nitzschia* spp. cells were seen at both beaches (September 22–23). Wind reversal immediately before arrow is on September 16–19.

through the summer months. Upwelling favorable winds were sustained from late August through early September, and then began to weaken in strength toward the end of September. In the beginning of October, a strong transition to northward (non-upwelling) winds was observed.

A decrease in water temperature was recorded at both beaches in mid-August, followed 1 week later by a warming event, which corresponded to the first observation of toxic *Pseudo-nitzschia* spp. (Fig. 5b). Water temperature decreased by approximately 6.0 °C at Kalaloch Beach and 4.5 °C at Second Beach from late August to mid-September, coincident with strong northwesterly winds. Water temperature increased at both sites during the last 2 weeks in September and into early to mid-October. At both beaches, the maximum numbers of *Pseudo-nitzschia* spp. cells were seen during this temperature increase (solid arrow).

Pseudo-nitzschia pseudodelicatissima Growth Study

Pseudo-nitzschia pseudodelicatissima in culture had apical axes of 38–60 μm, transapical axes of 2–2.5 μm, 33–40 striae in 10 μm, 18–21 fibulae in 10 μm, and 5–6 poroids per 1 μm. Except for the apical axes, which were approximately half of the reported values, these measurements are similar to those describing *P. pseudodelicatissima* in Hasle et al. (1996). Figure 6 shows average growth rate and DA production for duplicate cultures of *P. pseudodelicatissima*. DA activity increased steadily through exponential growth phase, and as the cells reached the late stationary growth phase, DA production dramatically increased. This experiment illustrates that an isolate of *P. pseudodelicatissima* from Washington coastal waters does produce DA as measured by a receptor binding assay, and toxin production increases as the cells reach stationary phase.

DISCUSSION

Domoic Acid Observations on the U.S. West Coast in 1998

The same species of *Pseudo-nitzschia* was not responsible for all toxic events along the U.S. west coast during 1998. DA was first detected in sardines and anchovies in California coastal waters in May 1998, then subsequently measured in razor clams on the Oregon coast beginning in late July and in Washington State razor clams later that summer. *Pseudo-nitzschia australis* was responsible for the death of over 50 sea lions in May and June 1998 along the central California coast (Gulland et al. 1999, Lefebvre et al. 1999, Scholin et al. 2000). Levels of DA in razor clams in Oregon, above the 20 μg/g regulatory limit, coincided with the presence of *P. australis* in coastal waters beginning in late July (D. Cannon pers. comm., Trainer et al. in press.). In contrast, we report here that the toxic event on the Washington coast that resulted in record levels of DA in razor clams in October 1998 was attributable to a bloom of *P. pseudodelicatissima*.

The association of different *Pseudo-nitzschia* species with distinct toxification events indicates that the hydrographic factors influencing toxic bloom occurrence and toxic cell transport may be unique to a given U.S. West Coast region. Given the southward surface flow of the California current system and maximum Columbia River discharge as a low salinity, low density, offshore plume lying to the southwest during summer months, it is unlikely that a northward transport of phytoplankton cells is a primary means by which toxic cells are spread northward along the coast from California to Washington. We hypothesize that onshore advection of localized offshore populations of *Pseudo-nitzschia* spp. cells that are present in U.S. West Coast waters during summer months (Horner et al. in press) is the likely means of toxic bloom initiation in Washington State waters.

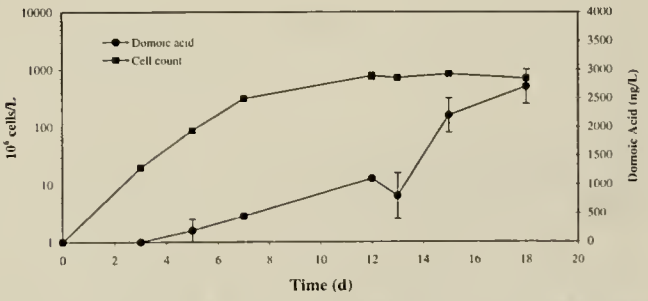

Figure. 6. Cell counts and domoic acid levels in a cultured isolate of *P. pseudodelicatissima*. The error bars indicate the range of values from duplicate measurements at each time point.

Environmental Conditions

What were the environmental conditions that allowed a toxic bloom to occur? Observations of toxic blooms in eastern Canada, central California, and Puget Sound, Washington, have indicated that rainfall may provide a significant source of nutrients that are important in bloom initiation. High precipitation following an unusually dry summer was suggested as a causative factor in 1987 DA incident in eastern Canada (Bird and Wright 1989, Smith et al. 1990), with runoff supplying the nitrogen source. Similar conditions were documented in the 1991 DA episode off the Washington coast, which occurred after a hot, dry period followed by rain (Horner and Postel 1993). Lack of runoff in central California during spring through autumn is the norm (Buck et al. 1992); however, record levels of rainfall were experienced in central California during the early months of 1998 before the outbreak of *P. australis* (Trainer et al. 2000, Scholin et al. 2000). Rainfall and corresponding river runoff, followed by calm weather, were important factors in the development of a *Pseudo-nitzschia* spp. bloom in Penn Cove, Puget Sound in the summer of 1997 (Trainer et al. 1998).

Rainfall, however, is not the only source of nutrients to coastal environments. The small upwelling events that punctuate the oceanic autumn/winter transition in the northeastern Pacific (Bolin and Abbott 1963) may substitute for freshwater runoff as a nitrogen source. The toxic *P. australis* blooms observed off the California coast in 1998 were positioned in coastal upwelling zones (Trainer et al. 2000). The present study shows that nutrient inputs from upwelling in the absence of significant rainfall fueled the *Pseudo-nitzschia* spp. bloom off the central Washington coast in late September 1998. These case histories make it clear that a pulse of nutrients, whether from rainfall and subsequent river runoff or from coastal upwelling events, is required to fuel toxic *Pseudo-nitzschia* spp. blooms.

The transition to wind relaxation and reversal events in mid- to late September and a strong reversal in early October, which marked the beginning of the autumn transition, resulted in the Ekman transport of surface water toward the coast beginning in mid-September. When upwelling winds reverse direction or relax, surface waters can be transported rapidly into the nearshore region where algal cells can mix vertically to the bottom (Donaghay and Osborn 1997). The beginning of the autumn transition in 1998 corresponded with a dramatic increase in water temperature at Kalaloch Beach (+ 4 °C) and at Second Beach (+ 2 °C; Fig. 5b). The subsequent relaxation of upwelling winds (Fig. 5a) corresponded to an increase in the number of cells observed at both beach sites, reaching a maximum on September 22–23 (Fig. 4a,b).

Difference between Kalaloch Beach and Second Beach

Although species composition and cellular levels of DA were similar at both beaches during the course of this study, the maximum number of *Pseudo-nitzschia* spp. cells, specifically *P. pseudodelicatissima*, measured at Kalaloch Beach was at least three times higher than the levels measured at Second Beach. Our results suggest that a pulse of nutrients, especially nitrate, rather than a sustained nutrient supply could account for the difference in the intensity of the bloom and, thus, the total levels of DA in the seawater. Pulses of nitrate, possibly from resuspended sediments after wind events or from river runoff have been previously associated with blooms of *P. multiseries* in eastern Canada (Smith et al. 1990). The highest concentration of nitrate at Kalaloch Beach was measured on September 1, a significant increase (175-fold) over levels recorded on August 25. Although nitrate levels increased slightly on September 1 at Second Beach, the change in concentration of this nutrient was not significant through September. Chlorophyll *a* levels were also routinely higher at Kalaloch Beach than at Second Beach (Fig. 4c). This clearly indicates that hydrographic factors at Kalaloch Beach were more supportive of phytoplankton blooms in the summer and early autumn months. The physical oceanography that explains the different productivity at Kalaloch Beach compared with Second Beach will be detailed in future studies.

Toxigenic Pseudo-nitzschia pseudodelicatissima

Our measurement of increasing DA levels in a cultured isolate of *P. pseudodelicatissima*, and confirmation of DA by mass spectroscopy of field samples consisting of 90–100% *P. pseudodelicatissima*, show that this species is a DA producer in Washington coastal waters. Significant numbers of other known toxigenic *Pseudo-nitzschia* species were not found in field samples collected before and during the razor clam toxification event in 1998. The 1998 coastal event was not the only time that *P. pseudodelicatissima* has been linked to toxin production in Washington waters. This organism was the primary *Pseudo-nitzschia* species seen in offshore areas of DA production during cruises aboard the R/V McArthur in the summers of 1997 and 1998 (Horner et al. in press). However, *P. pseudodelicatissima* does not produce toxin in all areas of the world. For example, DA has not been measured in seawater at times when *P. pseudodelicatissima* formed dense blooms in Monterey Bay (Walz et al. 1994, Scholin et al. 2000) but it is known to produce toxin in the Bay of Fundy (Martin et al. 1990), in the Gulf of Mexico (M. Parsons pers. comm.), and in certain clones isolated from Danish waters (Lundholm et al. 1997). Genetic variability or differences in gene expression in various strains of *P. pseudodelicatissima* may explain the variation in toxin production by this organism in different regions.

Indeed, *P. pseudodelicatissima* did not produce measurable amounts of toxin at all times in both cultured and field samples. For example, it was observed at Kalaloch Beach on August 19 (Table 1) when no toxin was measured in seawater. Laboratory cultures showed that this alga produces maximum amounts of toxin in late exponential and stationary phases of growth. The highest cellular levels of DA in field samples were measured in early October as the bloom was declining. Therefore, to predict accurately the levels of toxin that will be produced by a bloom, some knowledge of the growth stage of the bloom population must be obtained. In the field, maximum numbers of *P. pseudodelicatissima* were observed at Kalaloch Beach about 2–3 weeks after a pulse of nutrients was recorded. Similarly, the cultured isolate reached stationary phase of growth in approximately 12 days. Perhaps similar growth rates and dependence of toxin production on growth stage can be expected in field samples compared to laboratory cultures, a possibility that needs to be determined empirically in future studies. This information will assist in the complete characterization of natural blooms of toxic *Pseudo-nitzschia*.

Pseudo-nitzschia spp. cells were present in nearshore waters through most of the summer at both beaches, indicating that they may be able to bloom during much of the year, given the appropriate environmental conditions. During most of the summer of 1998, nontoxic or weakly toxic species such as *P.* cf. *heimii* and *P.*

pungens, respectively, were present at times when DA was not detectable in the seawater. When a rich supply of nutrients became available, toxic *P. pseudodelicatissima* cells increased in number and reached bloom proportions (over 10^6 cells/L) over a 2–3 week period. It is not clear which specific environmental conditions caused *P. pseudodelicatissima* to become dominant and not any of the other species of *Pseudo-nitzschia* that were present in the assemblage during the summer months.

Prediction

The influx of nutrients in early September 1998 was a result of one of the strongest coastal upwelling events on the Washington coast that season as evidenced by persistent northwest winds (Fig 4a). Although toxic blooms in Monterey Bay occur in the spring and autumn, the Washington coast has recorded DA events primarily in autumn months. This observation makes toxic blooms in Washington potentially more predictable and perhaps more strongly linked to nutrient pulses during coastal upwelling events, especially in years of low rainfall. If nutrient concentrations and environmental conditions are to be used as predictive factors, more in-depth studies of these parameters as they relate to the composition of the phytoplankton assemblage must be undertaken.

Close observation of the developing *Pseudo-nitzschia* population during late summer and early autumn months and daily monitoring of environmental factors such as upwelling indices could provide an early warning of toxic blooms that affect coastal razor clam populations. Molecular probes able to detect toxic *Pseudo-nitzschia* species found seasonally in Washington coastal regions, possibly in the form of automated sensors placed on buoys, could warn recreational and subsistence shellfishers of an impending bloom event (Scholin et al. 1999). Although we know that razor clams can become toxic within 18 days of the appearance of a toxic bloom, the precise timing between the appearance of high levels of DA in seawater, and the accumulation of toxin in clams must still be determined in order to fine tune our predictive capabilities.

CONCLUSIONS

The late summer/early autumn outbreak of toxic *Pseudo-nitzschia* was strongly linked to the relaxation of seasonal upwelling in a year of unusually low rainfall. The bloom occurred at a time when winds reversed direction, allowing cells to be brought to the coast. This scenario presumes an established, offshore population of *P. pseudodelicatissima*, of which recent research cruises provide evidence (Horner et al. in press, Trainer et al. in press). We conclude that the pennate diatom *P. pseudodelicatissima* was the major source of DA on the central Washington coast in the late summer and autumn of 1998, with record levels of toxin in razor clams detected in October of that year. Our study illustrates that toxic *Pseudo-nitzschia* can, indeed, contribute to the surf-zone diatom community on which razor clams feed. Natural depuration of razor clams was still not complete as of October 1999. This slow depuration of toxin from razor clams has made a further impact on the already depressed coastal economies of Washington State by necessitating a second season of beach closures likely resulting from one toxic bloom.

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A CASE OF CONSISTENT SPATIAL DIFFERENCES IN CONTENT OF DIARRHETIC SHELLFISH TOXINS (DST) AMONG THREE BIVALVE SPECIES: *MYTILUS EDULIS*, *OSTREA EDULIS*, AND *CERASTODERMA EDULE*

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ABSTRACT Content of diarrhetic shellfish toxins (DST) was compared among mussels (*Mytilus edulis*), oysters (*Ostrea edulis*), and cockles (*Cerastoderma edule*) at two spatial scales: regions (100 km apart) and locations within regions (5 km apart). Samples were analysed for DST using protein phosphatase inhibiton assay in individual digestive glands. Concentrations of DST in all oysters and cockles were below the detection limit in the assay, whereas mussels from both regions and all locations contained mean levels of DST above the regulation limit for harvest and marketing. Thus interspecific differences in content of DST were found along the Swedish west coast. Some behavioral and physiological phenomena are proposed to explain the differences among species. These include differential uptake and processing of toxic algae, biotransformation of toxins, and reduced filtration at low temperatures. These findings may have some implications for harvest and cultivation of bivalves and suggest a possibility that cockles and oysters could be marketed for human consumption during periods of elevated levels of DST in mussels.

KEY WORDS: *Cerastoderma edule*, cockles, diarrhetic shellfish toxins, DST, interspecific differences, *Mytilus edulis*, mussels, okadaic acid, OA, *Ostrea edulis*, oysters, protein phosphatase inhibition assay

INTRODUCTION

Diarrhetic shellfish poisoning (DSP) is one of several illnesses caused by the consumption of shellfish containing toxic substances produced by marine microalgae. The most common toxins associated with DSP belong to the okadaic acid (OA) group (Yasumoto et al. 1985). DSP was first described in Japan during the late 1970s, but has since been reported from temperate waters around all continents where it causes considerable problems for harvesters and consumers of shellfish.

In Sweden, monitoring for DSP toxins (DST) in blue mussels, *Mytilus edulis*, has been ongoing since 1988. This has revealed large seasonal, geographical, and individual variations in content of DST in mussels along the West Coast (Lindegarth 1997). For example, mussels generally contain DST above the quarantine levels (160 μg OA kg^{-1} mussel meat, EC regulations) for harvest during the autumn and winter period, sometimes for up to 6 mo each year.

DST is the most serious threat to a sustainable industry based on cultured blue mussels in Sweden and there is a need for managerial actions to reduce its impact. Aquaculture and fisheries for a more diverse range of bivalve species, which may vary in content of toxins, could be an alternative during periods of high levels of DST in mussels. As an example, the knowledge about interspecific differences in levels of paralytic shellfish toxins (PST) are used by authorities in Maine, who have been practising species-specific harvest and closure of bivalve fisheries for many years (Dr. S. Shumway pers. comm.). In Sweden, small-scale fisheries for the European oyster, *Ostrea edulis*, and cockles, *Cerastoderma edule* and *Ceratoderma lamarcki*, are in operation today. These species are currently not included in DST monitoring and information about the presence of DST in oysters and cockles is sparse.

As mentioned earlier, differential patterns of accumulation and depuration of PST among species of bivalves have been observed (Bricelj and Shumway 1998). In general, *Mytilus* sp. rapidly accumulate and detoxify PST compared to most other species. Some observations on differences in content of DST among species of bivalves collected in the field have also been made (MacKenzie et al. 1998, Poletti et al. 1998). *Mytilus* spp. were reported to contain higher levels of DST compared to the other species included in these studies. However, these studies were not specifically performed to investigate differences among species concerning their ability to accumulate DST. For that purpose shellfish should be collected at the same time and habitat to reduce effects of differences in exposure to toxic algae prior to sampling.

During the summer of 1998, high levels of OA (>1 mg OA kg^{-1} mussel meat) were detected in blue mussels from a farm located in north Bohuslän on the Swedish west coast. At the same time, we

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observed that oysters and cockles, collected from the vicinity of the farm, contained non-detectable levels of DST measured by HPLC. To test whether the observed differences in DST among these species were real and consistent in space, we sampled at two different spatial scales. This paper reports the results from this study.

MATERIALS AND METHODS

Naturally occurring cockles (*Cerastoderma edule*), oysters (*Ostrea edulis*), and mussels (*Mytilus edulis*) were collected during 1 wk in late November to early December 1998 from two regions separated by approximately 100 km along the coast of Bohuslän County, Sweden (A: Ljungskile and B: Tjärnö, Fig. 1). In these regions mussels are commercially farmed and fishing for both cockles and oysters occurs. Sampling was performed when levels of OA in farmed mussels had been reported to be high for more than 2 mo in both areas (OA data from the weekly monitoring program). Within each region sampling was done at each of two locations, separated by approximately 5 km (Buvenäs and Sparreviken in region A, Tenskär and Kockholmen in region B, Fig. 1). These locations were selected because all three species were found to co-exist on a small scale. Six individuals of each species were taken from each location at depths between 0.5 and 3 m within a radius of 20 m. Sampling depths and size characteristics of the samples from each location are shown in Table 1. Water temperatures were between 3 °C and 5 °C at the time of collection. The specimens were frozen and stored at −20 °C until toxin analysis was performed.

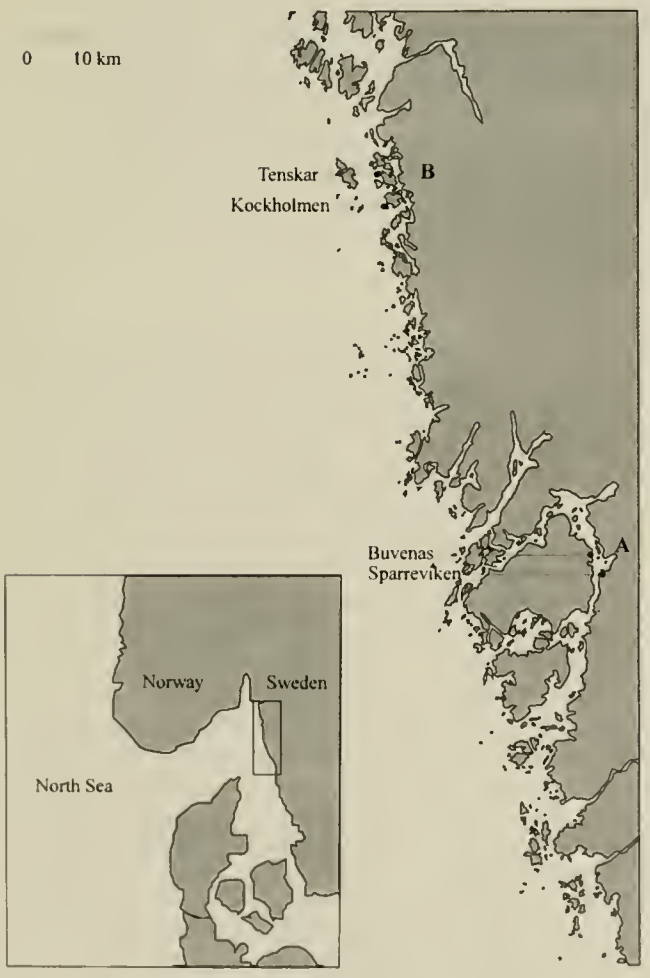

Figure 1. Map of the coastal area of Bohuslän County showing the sampling locations of *M. edulis*, *O. edulis*, and *C. edule*. (A) Ljungskile region. (B) Tjärnö region.

Preparations of shellfish extracts were done on individual digestive glands using the sample clean-up protocol according to Lee et al. (1987). Concentrations of DST in the resulting chloroform extracts were then analyzed using the fluorescent microplate phosphatase inhibition assay (PIA) according to Vieytes et al. (1997) with some modifications which will be published elsewhere (Rehnstam-Holm et al. in prep.). Toxin content was expressed as micrograms of OA equivalents g^{-1} of digestive gland and micrograms of OA equivalents kg^{-1} mussel meat. The detection limit for shellfish extracts was 5 ng OA equivalents g^{-1} digestive gland when dilution factors were considered.

To confirm the accuracy of the PIA method, 4 individuals of each species were chosen at random and analyzed for OA and DTX-1 by HPLC according to Lee et al. (1987) using 1-pyrenyldiazomethan (PDAM) instead of ADAM.

RESULTS

Results from the PIA analyses are shown in Table 1. All the cockle and oyster samples contained levels of DST below the detection limit for the PIA, whereas toxins were detected in all individual mussels. Mean concentration in mussels varied between 1.5 to 2.6 μg OA equivalent g^{-1} digestive gland or 209 to 241 μg OA equivalent kg^{-1} mussel meat which is above the regulation limit for marketing of mussels.

When analysed by HPLC, neither OA nor DTX-1 were detected in the oyster and cockle extracts, whereas OA was detected in all mussel samples. Low amounts of DTX-1 compared to OA (>10%) were found in two of the mussel samples. OA equivalent measured by the PIA method correlated well to concentrations of OA in the HPLC method (data not shown). Thus HPLC confirmed the results from the PIA that OA and DTX-1 were absent (non-detectable) in the oyster and cockle samples.

This study was designed to test hypotheses about interspecific and spatial variability in DST using multifactorial ANOVA. Since no toxins were detected in *C. edule* and *O. edulis*, tests with normal parametric procedures could not be justified in order to test hypotheses about interspecific differences in mean content of DST. Nevertheless, confidence intervals did not include the value for detection limit for the PIA which indicated that the sample size (n = 6) was sufficiently large to allow unambiguous conclusions about interspecific differences.

DISCUSSION

The purpose of this study was to investigate interspecific and spatial variability of DST in 3 bivalve species that co-exist in Swedish waters. To our knowledge this is the first study where levels of DST have been compared among bivalve species that were sampled from the same locations under similar conditions in the field. The consistent differences in levels of DST found between blue mussels on one hand and cockles and oysters on the other hand provided evidence for interspecific differences that are not caused by differences in exposure to toxic algae. Thus the pattern that was found indicated differences in either the ability to accumulate or depurate DST among the species studied.

TABLE 1.

Sampling depths, size characteristics, and content of OA, analyzed by PIA in *C. edule*, *M. edulis*, and *O. edulis* from the 4 study locations. % d.g.: proportion digestive gland (%) of total tissue wet weight. Values for size characteristics are means ± SD (n = 6). Concentration of OA is expressed as µg OA equiv. g^{-1} digestive gland and µg OA equiv. kg^{-1} mussel meat. n.d.: no toxins detected (detection limit 5 ng OA equiv. g^{-1} digestive gland). Values for OA are means ± 95% confidence intervals (n = 6).

Species	Region	Location	Depth (m)	Shell length (mm)	Tissue wet weight (g)	% d.g.	OA equiv. (µg g^{-1})	OA equiv. (µg kg^{-1})
C. edule	Ljungskile	Buvenäs	0.5–0.7	40.0 ± 5.7	10.4 ± 4.3	7.6 ± 1.2	n.d.	
		Sparreviken	0.5	44.5 ± 4.6	13.0 ± 2.5	8.4 ± 1.8	n.d.	
	Tjärnö	Kockholmen	0.5–0.7	40.5 ± 2.7	9.3 ± 1.8	11.5 ± 3.2	n.d.	
		Tenskär	0.6–1.0	26.8 ± 9.2	3.4 ± 2.7	11.4 ± 1.4	n.d.	
M. edulis	Ljungskile	Buvenäs	0.5–0.7	63.2 ± 7.9	17.5 ± 7.0	8.6 ± 1.8	2.6 ± 1.4	222 ± 119
		Sparreviken	3.0	73.5 ± 7.2	29.9 ± 9.0	9.6 ± 2.0	2.4 ± 2.0	242 ± 223
	Tjärnö	Kockholmen	1.7–2.2	74.5 ± 14.7	29.2 ± 14.3	14.8 ± 3.2	1.4 ± 0.5	209 ± 92
		Tenskär	0.6–1.0	57.3 ± 14.3	16.6 ± 11.2	13.4 ± 2.9	1.5 ± 0.5	210 ± 105
O. edulis	Ljungskile	Buvenäs	0.5–0.7	71.3 ± 13.0	10.8 ± 6.1	9.2 ± 2.7	n.d.	
		Sparreviken	3.0	80.3 ± 15.5	20.0 ± 10.4	9.1 ± 2.2	n.d.	
	Tjärnö	Kockholmen	1.7–22	91.5 ± 8.8	19.7 ± 8.5	8.2 ± 1.8	n.d.	
		Tenskär	0.6–1.0	54.3 ± 5.9	2.7 ± 1.2	11.3 ± 3.6	n.d.	

To explain the interspecific differences in content of DST, some behavioral and physiological phenomena can be proposed as discussed below.

Interspecific variability in pre- and/or post-ingestive selection may occur in order to increase or reduce the uptake and processing of the DST-producing algae. *O. edulis* from the North American east coast has been found to selectively clear both toxic (PST) and non-toxic dinoflagellates from mixed cell suspensions in laboratory experiments (Shumway and Cucci 1987). Sidari et al. (1998) observed that during an event of DSP in Italy, *M. galloprovincialis* seemed to feed selectively on *Dinophysis* sp., comparing algal contents in the stomachs to those of the water column. Although there are no previous observations on selection against algae containing DST, a possible explanation of our results is that the sampled populations of *O. edulis* and *C. edule* may be rejecting these species, either pre- or post-ingestion, thus rendering the non-toxic results in the analysis.

Recent evidence suggests that DST can be biotransformed by bivalves (Lee et al., 1989, Suzuki et al. 1999, Fernández et al. 1996). A group of low-polar acyl-ester derivatives of OA, DTX-1 (referred to as DTX-3), and DTX-2 has been found in shellfish, but never in the dinoflagellates producing DST. Therefore, it has been suggested that the acylated forms of DST are products of metabolic activity in the digestive glands of the molluscs (Lee et al. 1989). Using modifications of the HPLC method, Suzuki et al. (1999) reported that Japanese scallops, *Pactinopecten yessoensis*, rapidly converted DTX-1 to DTX-3 with significantly higher content of the latter in the tissue. In contrast, Fernández et al. (1996) found that although mussels, *M. galloprovincialis*, contained detectable amounts of acylated DST, the major content in the extracts was always OA, implying only a slow rate of biotransformation of OA for mussels. Due to the chemical nature of the acyl derivatives, they are not detected using traditional clean-up procedures for HPLC. Also, the sensitivity of the PIA method to DTX-3 are low compared to OA and DTX-1 (Mountfort et al. 1999). Thus the absence of OA and DTX-1 in oyster and cockle extracts in our study could be explained by a rapid acylation of these compounds in the digestive glands, which in that case, we were unable to detect by PIA and HPLC. In future studies it should be possible to test this hypothesis by using modifications of the HPLC method. It is also possible that rates of depuration of the non-acylated compounds vary among species, which could be evaluated by performing depuration experiments.

The interspecific differences in content of DST could also be an effect of water temperature since sampling was performed during a time of the year when temperatures were low (3 °C–5 °C). This is close to the temperature when filtration is reduced or inhibited in oysters (Child and Laing 1998). Mussels, on the other hand, are active even at temperatures close to 0 °C (Loo 1992). Reduced filtration and thus uptake of toxic algae in oysters and also cockles may therefore explain the results. Also, in case that these species did contain toxins earlier during the season, depuration may have occurred after filtration (uptake of toxic algae) stopped.

Although the mechanisms causing the interspecific differences in content of DST remain unknown, the observations made in this study could have important implications for harvest and cultivation bivalves. Currently within the EC, marketing of shellfish from certain areas is regulated by content of algal toxins in blue mussels, a species which generally accumulates high levels of toxins. Our results suggest the possibility that oysters and cockles could be harvested and marketed for human consumption during periods when mussels contain significant levels of toxins. However, further sampling and analysis of DST in oysters and cockles, including temporal replication must be performed during periods of DST in order to confirm the results found in this study. Also, biological tests should be conducted in parallel with the chemical analyses to test whether any toxicity due to unknown substances or biotransformed products is present in the shellfish meat.

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JOURNAL OF SHELLFISH RESEARCH

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CONTENTS

CONTENTS CONTINUED ON INSIDE BACK COVER